CHARACTERIZATION OF AUTOCLAVED FLAXSEED AS FEED FOR RUMINANTS USING CONVENTIONAL AND MID-IR SPECTROSCOPIC BASED APPROACHES

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ABSTRACT

The objectives of this study were to investigate the effects of autoclave heating on the rumen protein degradation characteristics of flaxseed (*Linum usitatissimum*, cv. Vimy), and to compare them to differences in diffuse reflectance infrared Fourier transform (DRIFT) and Synchrotron based Fourier transform infrared microspectroscopy (S-FTIR) measurements of the protein α-helix to β-sheet ratios. Hierarchical cluster analysis (CLA) and principal components analysis (PCA) were also conducted to identify differences in the DRIFT spectra. Flaxseed samples were kept raw for control or autoclaved in batches at 120°C for 20, 40 or 60 min for treatments 1, 2 and 3, respectively. The rumen degradation kinetics of protein were measured along with the protein sub-fractions of the Cornell net carbohydrate and protein system (CNCPS), and chemical composition. Intestinal digestibility was determined using the three-step procedure outlined by Calsamiglia and Stern (1995). Protein supply to the small intestine was determined using the NRC (2001) and DVE/OEB models. The results showed that heating increased dry matter (DM) and ether extract (EE) content, while reducing neutral detergent fibre (NDF) and acid detergent fibre (ADF), with little numerical difference between the three treatments. Soluble crude protein (SCP) also decreased upon autoclaving with concomitant increases in non-protein nitrogen (NPN), neutral detergent insoluble nitrogen (NDIN) and acid detergent insoluble nitrogen (ADIN). The CNCPS protein sub-fractions with the greatest changes were the buffer-soluble true protein fraction (PB1) and the fraction representing buffer-insoluble true protein which is not bound to NDF (PB2) showing dramatic increases, indicating a decrease in the overall protein degradability. *In situ* experiments showed a reduction in effective degradable dry matter (EDDM) as well

as a reduction in effective degradable crude protein (EDCP) without significant differences between the treatments. Intestinal digestibility of protein as estimated by the three-step procedure showed no changes upon autoclaving. Modeling results, with flaxseed as the only feed source, for absorbable ruminally-undegraded feed protein in the intestines using both the NRC (2001) and DVE/OEB systems showed increases as a consequence of the autoclave treatments but again there were no differences between the treatments. The degraded protein balance results showed for both the NRC (2001) and DVE/OEB models that both were decreased upon autoclave treatment. However, the values for the NRC (2001) model suggested a potential nitrogen (N) deficiency and, therefore potentially impaired microbial crude protein (MCP) production, whereas the values for the DVE/OEB system showed potential N excess and, therefore, possible loss from the rumen. DRIFT analysis of protein secondary structure ratios showed a decrease in the α-helix to β-sheet ratio for the whole seed, whereas results from S-FTIR spot data for cotyledon tissue showed autoclaving had the opposite effect on the ratio. CLA and PCA were successfully used to make distinctions between the different treatment spectra and showed enhanced sensitivity upon selection of a smaller spectral window to include only the amide I and II portion of the IR spectrum. The results failed to demonstrate any differences between the autoclave treatments used in this study, and showed that autoclaving generally decreased effectively ruminal degradability of flaxseed protein. The results further indicated that autoclaving had a significant enough effect on the flaxseed to permit identification of the altered α-helix to β-sheet ratio with the mid-IR spectrum, as well as differentiation between the treatments using PCA and CLA. PCA

and CLA results suggest that mid-IR spectral methods are more sensitive than traditional methods when used to identify differences between the heat treatments.

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"To get through the hardest journey we need take only one step at a time, but we must keep on stepping." Chinese Proverb

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LIST OF ABBREVIATIONS

ABCP	Absorbable bypass crude protein
ADF	Acid detergent fibre
ADIN/ADIP	Acid detergent insoluble nitrogen (ADIP = ADIN * 6.25)
ADL	Acid detergent lignin
AECP	Absorbable endogenous crude protein
AMCP	Absorbable microbial crude protein (in NRC-2001 model)
AMP	Absorbable microbial crude protein (in DVE/OEB system)
ARUP	Absorbable ruminally undegraded protein
B	Bypass fraction
BCP	Bypass crude protein
BPS	Bacterial protein synthesis
C	Carbon
CHO	Carbohydrate
CLA	Hierarchical cluster analysis
CNCPS	Cornell Net Carbohydrate and Protein System
CP	Crude protein
D	Degradable fraction
DM	Dry matter
DPB	Degraded protein balance
DVE	Protein absorbable in the small intestine
DRIFT	Diffuse reflectance infrared Fourier transform spectroscopy
dRUP	Digestibility of ruminally undegraded feed protein
ED	Effectively degradable fraction
E_MP	Potential microbial protein synthesis from the energy gained during anaerobic fermentation
EE	Ether extract
ENDP	Endogenous protein
FOM	Fermentable organic matter

FP	Fermentation product content of ensiled feeds
FSD	Fourier self-deconvolution
H	Hydrogen
h	Hours
IIP	Indigestible intake protein
IR	Infrared
K_d	Degradation rate
K_p	Passage rate
min	Minutes
MP	Metabolizable protein
N_MP	Potential microbial protein synthesis from ruminally degradable feed protein
N-3	Omega 3 fatty acid
N-6	Omega 6 fatty acid
NDF	Neutral detergent fibre
NPN	Non protein nitrogen
O	Oxygen
OEB	Degradable protein balance from Dutch system
PA	Protein sub-fraction A described in CNCPS
PB1	Protein sub-fraction B1 described in CNCPS
PB2	Protein sub-fraction B2 described in CNCPS
PB3	Protein sub-fraction B3 described in CNCPS
PC	Protein sub-fraction C described in CNCPS
PCA	Principal components analysis
RUP	Ruminally undegradable protein
Sec	Seconds
S:N	Signal to noise ratio
SCP	Soluble crude protein
S-FTIR	Synchrotron based Fourier transform infrared spectroscopy
TDN	Total digestible nutrients

U	Undegradable fraction
UDM	Undigested dry matter
VRAS	Digestible inorganic matter

1. INTRODUCTION

1.1 Flaxseed

Historical records show the cultivation and use of flax as early as 3000 B.C. Throughout history, the health benefits of flaxseed have been extolled by the likes of Hippocrates and Charlemagne. Since the cultivation of this crop began, other uses for the plant have emerged and include clothing (linen), flooring (linoleum), and the many industrial uses for its oil (paint, varnish, rust inhibitor). More recently, the value of flaxseed has again been recognized in terms of promoting good health due to the high omega-3 fatty acid content of its oil (Flax Council of Canada, 2007a).

Canada is the world's largest producer and exporter of flaxseed (Flax Council of Canada, 2007b). Within Canada, Alberta, Saskatchewan and Manitoba, are the major producers, with Saskatchewan being the largest producer by far (Flax Council of Canada, 2007c). In terms of composition, flaxseed oil is sensitive to climate. The cooler climate that Canadian flaxseed is grown in, for example, results in increased oil content with α-linolenic acid (ALA) making up about 9% more of the oil when compared to flaxseed grown further south in the United States (Bhatty 1995).

A typical North American's diet lacks polyunsaturated fatty acids. This situation could be improved through dietary inclusion of flaxseed (Kris-Etherton et al. 2000). The benefits are derived primarily from the oil it contains, specifically the content of ALA which is a precursor to bioactive compounds in the human body and in livestock animals. The physiological systems that ALA plays some role in are varied and include the immune

system, central nervous system, visual development and the cardiovascular system (Kelly et al. 1991; Delion et al. 1994; Neuringer and Connor, 1986; Allman et al. 1995).

The potential benefits of using flaxseed as a feed necessitate a greater understanding of flaxseed in terms of nutrition in ruminants. Flaxseed on average contains 26% protein on a moisture-free basis, whereas solvent-extracted flaxseed meal consists of 44% CP on a moisture-free basis (Bhatty 1995). The protein content of flaxseed is sensitive to growing location and soil conditions and shows a negative correlation with oil content. Furthermore, as protein content increases so too does the protein solubility, which suggests a change in protein quality (Oomah et al. 1995). Most flaxseed breeding programs at this point are concerned with oil content and quality rather than protein. More work is needed to define flaxseed protein with respect to nutrition.

The real potential for the feed industry is the production of healthier animals that would be a source of healthier products for human consumption. The challenge is to define the nutritional qualities of flaxseed as a feed source for dairy and cattle production and to develop and promote improved human and livestock health through traditional production systems. As consumption increases, more meal and by-products should become available, providing industry with a readily available protein source for ruminants. Studies, however, typically have focused on flaxseed oil and its ALA content. Canada, the world's largest producer of flaxseed, stands to benefit from the development of flax as a more common feed ingredient and to take advantage of the opportunities that will arise from the quality of the flaxseed it produces.

1.2 Why Synchrotron based FTIR?

Structural considerations are important when evaluating digestibility in the rumen (Jung and Allen, 1995; Akin 1988). There are multiple levels of structure involved in understanding how digestibility may be influenced by treatments such as heating. The first is the physical structure of a feed, which depends on which part of the plant is being fed. The physical structure could be seeds, as in the case for feeding grains, or it could be leaves and stems, in the case of feeding hay. In either case, there are physical barriers, inherent to the feed, which prevent the digestion process from proceeding and include features like the seed coat which must be overcome for the grain to be digested. Rumination is an example of an adaptation where additional chewing assists in breaking down the feed. Processing of the feed may alter these structures as well (Yang et al. 2000). A second level of structure that influences degradability pertains to inter-nutrient interactions or biological component matrices. It entails the associations between starch, protein, fibre, lignin, fat and other components of the feed. These interactions are essential for the plant to survive and consist of oil bodies and starch granules which are closely associated with storage proteins. The interactions between two or possibly more nutrients may act as a barrier to digestion. In the case of starch granules, the interaction of protein with starch affects the rate at which starch may be degraded by rumen microorganisms (McAllister et al. 1993). Lignin associated with fibrous components may also prevent the rumen microbes from fully degrading the fibrous component of feed (Chesson 1988). Another level of structure is that of the nutrients themselves, in other words, the macromolecular arrangement of the nutrients. In a starch molecule, for example, this would be affected by the number of branches and the length of those

branches. Each branch point, for example, consists of an α-1,6-linkage between glucose moieties and requires enzymes other than α-amylase, such as pullulanase or iso-amylase, for hydrolysis (Kotarsky et al. 1991), thus providing some other barrier to digestion. In a protein molecule, the amino acid sequence affects the secondary structure of the protein, unlike for starch where all the subunits are glucose and bonds between subunits determine the structure of the starch molecule. Protein secondary structure will influence the location of peptide bonds as a result of protein folding, and some bonds would be tucked away in the interior of a protein. This presents the digestive enzymes with another obstacle to breaking protein down into absorbable peptides and amino acids. Finally, not all peptide bonds are broken by the same protease; the consequence of this is that protein primary structure determines which enzymes are required for complete breakdown of a protein.

Given this relationship of structure to digestion and digestibility, a new method shows promise as a means of feed evaluation. Synchrotron based Fourier transform infrared (S-FTIR) microspectroscopy is capable of generating molecular and structural information on the constituents of a feed at the sub-cellular level, while maintaining the physical orientation of the information (Wetzel et al. 1998; Budevska 2002). S-FTIR is sensitive to all of the previously mentioned levels of structure, the difficulty lies in interpretation of the data. Consequently, S-FTIR has been used to distinguish between varieties of barley (Valier vs. Harrington) known to have different degradation characteristics and different digestibilities (Yu et al. 2004a, 2004b). S-FTIR can also be used to detect more specific feed structural characteristics such as protein secondary structure, which is altered as a result of heat treatment (Yu et al. 2005). The method is further validated for use in the

feed industry by the ability it lends the investigator to focus on specific plant structures or cells within a sample of interest (Wetzel et al. 1998), thus maintaining a physical context for data interpretation. The challenge is to link the structural and molecular characteristics of feed samples to the degradability of nutrients in order to further supplement existing models with information that would make them better predictors of degradability.

1.3 Objectives of literature review

The purpose of the literature review which follows is to establish some of the pertinent background information on the potential of flaxseed as a feed ingredient and protein source in ruminant diets. It covers the effects of heat processing feeds and how improvements in digestibility, with respect to production, can be achieved with heating. It also highlights the basics of Synchrotron based and thermal IR sourced Fourier transform infrared (FTIR) spectroscopy for feed evaluation by highlighting the kinds of information each gathers and how the information can be pertinent to the feed industry and potentially improve current models used in feed evaluation.

2. REVIEW OF LITERATURE

2.1 Dietary inclusion of flaxseed in animal rations

2.1.1 Oilseeds in ruminant rations

Oilseeds and oilseed meals are commonly included in ruminant rations (Stake et al. 1973; Ward et al. 2002). Oilseed meals are striped of most of their fat content and are a good source of protein and fibre while whole oilseeds are typically a good source of energy as well as unsaturated fats. Recent interest in providing ruminants with a greater supply of dietary unsaturated fatty acids comes in part from a human health standpoint. There is an effort to ameliorate the fat profile of milk and meat, by decreasing the ratio of saturated fatty acids to unsaturated fatty acids, to foster better cardiovascular health. This is based on the fact that some of the unsaturated fats, as a consequence of ending up in the bloodstream after feeding, are deposited in fat deposits and milk of an animal. Unsaturated fats will eventually become saturated if in the rumen long enough through a process called biohydrogenation. Unfortunately, not all of the unsaturated fat will be absorbed as such, extensive biohydrogenation occurs in the reducing environment found in the rumen which partially negates the effort of trying to get unsaturated fats into the diet to begin with. When fed whole, the oil from an oilseed is expected to be protected against biohydrogenation, due to its physical structure, but results are contradictory (Keele et al. 1989; Scollan et al. 2001a, 2001b).

There are some considerations for dietary fat in ruminants, in particular the effects it has on the protozoa and bacteria in the rumen. One such effect is the suppression of protozoal activity, which decreases the number of protozoa in the rumen (Czerkawski et al. 1975).

Protozoal suppression can lead to an improvement in bacterial protein synthesis (BPS) because there are fewer protozoa engaged in breaking down the bacteria or other dietary constituents supporting a futile nitrogen (N) cycle of nutrient breakdown and resynthesis (Jouany 1996). Fats and oils also have effects on the bacteria in the rumen, which results in impairment of organic matter (OM) digestion, energy digestion, and bacterial protein synthesis (BPS), and is dependent on the concentration of fats present (Ikwuegbu and Sutton 1982). The interactions between fats and oils on the major rumen inhabitants and interactions among the inhabitants themselves make the effects of fat/oil dietary inclusion diverse and not always predictable. The reduction in protozoal activity, for example, would increase the efficiency of BPS yet at the same time the fats are believed to inhibit BPS in bacteria. The inhibition of BPS is due to the toxicity that the free fatty acids, released when triglycerides are degraded, exhibit towards some bacterial species within the rumen. The result is a shift in the bacterial population in the rumen (Broudiscou et al. 1988). Depending on the extent of either of these occurrences, different results would be expected. These changes to rumen function restrict the use of fats and oils as energy supplements in ruminant rations despite some of the benefits they might provide.

2.1.2 Flaxseed shape and morphology

Commercial flaxseed varieties,shown in figure 2.1, are varied in size and colour. On average they are 5mm long, 2.5 mm wide and 1.5 mm thick (Peterson 1958). Figure 2.2 shows a micrograph cross section of flaxseed. The seed cross section, taken from the center of the seed, shows the epiderm, known as mucilage, seed coat, and a thin layer of endosperm surrounding two cotyledons. The endosperm and the cotyledons have different protein solubilities (Painter and Nesbitt 1969) and oil compositions (Dorrell

Figure 2.1. Enlarged view of both yellow and brown flaxseed varieties.

(Credit: iStockphoto/Tim Pohl)

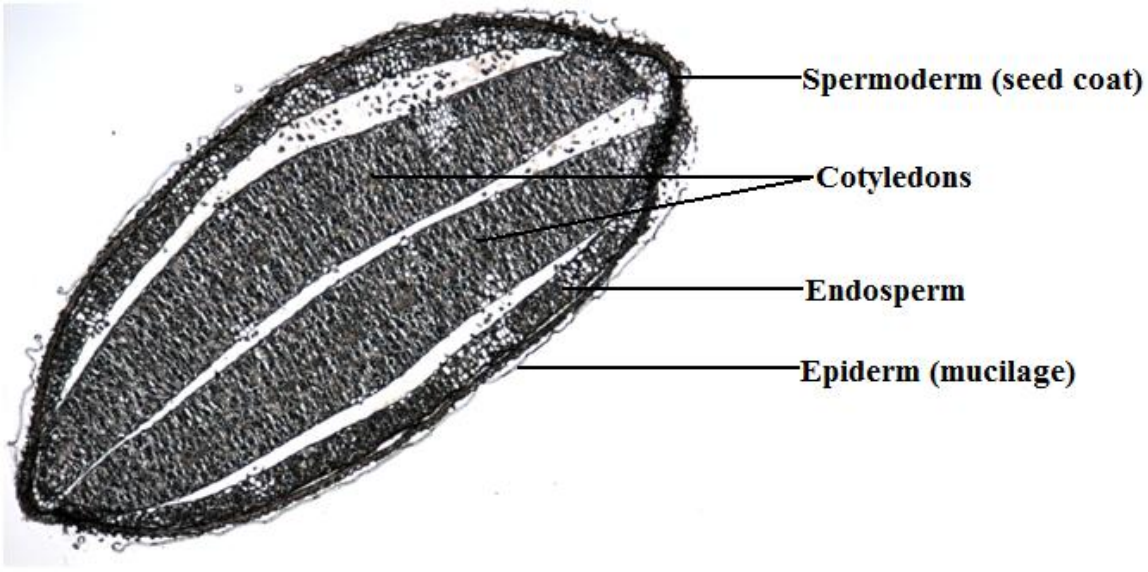

Figure 2.2. Cross section of a flaxseed.

1970). Not visible in Figure 2.2 is any discrete aleurone tissue. In the case of flaxseed, there are aleurone cells distributed throughout the endosperm and cotyledons. The shape of the aleurone grains within the aleurone cells differs between endosperm and cotyledon (Winton and Winton 1932). Oil is distributed throughout the endosperm and cotyledons. The oil is contained within the cells as microscopic droplets (Peterson 1958).

2.1.3 Flaxseed oil and protein characteristics

Canadian flaxseed contains, on average, 41% oil and 26% protein. Cultivar and growing conditions, however, play a significant role in the composition of flaxseed. Oil is a major component of flaxseed and it consists of several fatty acids, including palmitic acid (16:0) (5%), stearic acid (18:0) (3%), oleic acid (18:1n-9) (17%), linoleic acid (18:2n-6) (15%) and ALA (18:3n-3) (59%) (Bhatty 1995). ALA exhibits sensitivity to temperature post flowering, whereby the ALA content is favored by cooler post flowering temperatures at the expense of oleic acid content. It is of interest to note that the concentration of individual fatty acids in flaxseed oil is highly variable between seeds when environmental factors are equal (Bhatty 1995).

Flaxseed averages 26% protein on a moisture-free basis, while flaxseed meal consists of 44% CP on a moisture-free basis (Bhatty 1995). Similar to other oilseeds, the major storage proteins of flaxseed are albumins and globulins, which are classified based on their solubility in aqueous solvents. Globulins are the major storage protein and comprise roughly 58-66% of the total protein in flaxseed. Albumin is a much smaller protein in size and comprises around 20% of the protein content of flaxseed (Oomah and Kenaschuck, 1995). The secondary structure profile of the globulin storage protein

consists of 3% α-helix, 17% β-sheet, and 80% aperiodic (Madhasudhan and Singh, 1985a). The protein secondary structure profile of albumin is very different from that of globulin, and is comprised of 26% α-helix, 32% β-sheet, and 42% is aperiodic (Madhasudhan and Singh, 1985b). Considering the secondary structure profiles of the two major protein types, it is evident that that the predominant secondary structure, other than aperiodic, in flaxseed protein is the β-sheet. Oleosins are another important protein group found in flaxseed. Oleosins can make up 2-8% of total protein in oilseeds. The major function of oleosins is oil storage (Huang 1992). A large part of the function of these oil storage proteins is enabled by an elongated, anti-parallel β-sheet containing many hydrophobic residues. β-sheets make up about 30% of this protein in peanuts (Huang 1992). Oleosins are required for the formation of the oil bodies and, therefore, the storage of oil. These proteins, as a result of their amphipathic nature, are responsible for the oil/protein matrix of flaxseed which plays a role in protein and fat digestibility (Huang 1992).

2.1.4 Benefits of flaxseed feeding

Flaxseed shows promise as a feed ingredient for several reasons. One is the potential for improvements in herd health when flaxseed is added as a supplement to the diet. These improvements include a reduction in pregnancy loses as well as an increased conception rate that occurs when cows are supplemented with flaxseed (Ambrose et al. 2006). Other examples of improvement when supplementing cattle finishing diets, for example, are increases in performance and efficiency, as well as an increased marbling in meat (Maddock et al. 2006). These are possibilities that could both lead to increased profitability for the producer. However, there is still much work to be done with respect

to the effects of processing and how the other ingredients in the ration may interact with the flaxseed.

2.1.5 Dietary flaxseed and its effects

The fatty acids found in the diet are generally different than those absorbed by the small intestine. This is a result of the extensive biohydrogenation that occurs in the rumen and, generally speaking, the fatty acids on their way out of the rumen will be more saturated than those from the diet. The process of biohydrogenation can be avoided to some extent, however, by various chemical treatments, such as with formaldehyde or by heating or even by feeding whole oilseeds (Kennelly 1995). The ability to incorporate some flax-based fatty acids into milk and meat creates another opportunity for producers to create a value-added product with benefits to the consumer's health. In human nutrition, it is widely believed that modern western diets are deficient in n-3 fatty acids and too rich in n-6 fatty acids, and also contain much greater amounts of saturated fats than historic diets. The ratio of n-6 to n-3 fatty acids in the modern diet is typically around 20-30:1, whereas it is estimated that the ratio on which man lived throughout history is much lower, around 1-2:1 (Simopoulos 1999). There are various reasons for the shift in this ratio, but the consequences are clear with respect to human health, increased mortality from cardiovascular disease (Simopoulos 1999). The increased mortality is a result of the physiological effects that the n-6 and n-3 fatty acids produce. These physiological effects appear antagonistic to one another as a diet rich in n-6 fatty acids and low in n-3 fatty acids skews the physiological state to one that favors clotting, vasospasm and vasoconstriction, whereas the n-3 fatty acids are shown to have hypolipidemic, antithrombotic and anti-inflammatory effects (Simopoulos 1999). The ALA content of

flaxseed makes it an excellent source of n-3 fatty acids for the purpose of using dietary fats to modify milk and meat fat profiles. Scollan et al. (2001a) demonstrated that by feeding whole bruised flaxseed, they were able to modestly improve the n-6:n-3 ratio in both meat and adipose lipid profiles, while maintaining total muscle fatty acid content. Petit (2002) also demonstrated a similar shift in this ratio for milk fat from cows fed whole flaxseed. The implications for flaxseed as a feed ingredient are evident as it can rectify, to some extent, the fatty acid profile of the consumer's diet towards what the historical values would be. Combining this with growing consumer knowledge of functional foods and their ties to human health, flaxseed dietary supplementation should only increase in popularity as time passes.

2.2 Heat processing feeds

2.2.1 Means and purpose of heating feeds

Heat processing feeds is done in an effort to manipulate the digestive behaviour of the macronutrients contained within the feed. For protein, in terms of ruminant nutrition, the objective of heat treating a feed is to increase the amount of dietary protein that is passed out of the rumen, (bypass crude protein; BCP) without negatively affecting the digestibility of the protein in terms of the whole gastrointestinal tract (Yu et al. 2002). There are many ways to apply heat to a feed, but in general they are distinguishable by traits including the presence of moisture, as in autoclaving but not in toasting. They are also distinguishable in that some impart physical changes to the feed, such as extrusion or steam flaking. Changes in the rate and extent of rumen degradation depend on multiple factors, including the type of treatment used, the severity of the treatment in terms of time

and temperature, and the pH of the feed during heating. These changes are further complicated by the type of diet and the relative proportions of its constituents (Yu et al. 2002). There are several reactions that take place in a feed when heat is applied due to the diverse nature of the organic molecules that make up plant material comprising feeds. These reactions are chemical in nature and bonds are broken or formed whether they are, for example, covalent molecular bonds or non-covalent hydrogen bonds.

2.2.2 Heating and protein

As described by Yu et al. (2002), the primary means of altering the protein degradation characteristics upon heating is through denaturation. Denaturation of protein can occur at every level of protein structure. Quaternary structure is affected as heating breaks the non-covalent intermolecular bonds that hold the protein subunits together. Tertiary structure is affected because heating causes intramolecular non-covalent bonds and disulfide bonds to break, causing the protein to unfold. Secondary structure is affected by heat in a manner similar to tertiary structure. However, the non-covalent bonds involved are hydrogen bonds necessary to stabilize the α-helical and β-sheet conformations. Finally, primary structure may be altered by the breaking of the covalent bonds that hold the polypeptide chain together. The extent to which these changes occur is dependent on the severity of the heating conditions during treatment, not solely temperature but also moisture and pH. Typically, once the primary structure begins to be affected, the process is more appropriately termed protein degradation.

2.2.3 Heating and the Maillard reaction

One major chemical reaction that takes place when feedstuffs are treated with heat is the Maillard reaction. The Maillard reaction is responsible for a diverse range of chemical products that can influence the nutritional value of a food or feed (Martins et al. 2001). Among the most important processes of non-enzymatic Maillard reactions relates to the reaction of amino acids, peptides and proteins with reducing sugars and vitamin C (Arnoldie 2001). The Maillard reaction is dependent on the presence of reducing sugars such as glucose and fructose, both monosaccharides, but the reaction may also proceed in the presence of disaccharides such as lactose. Sugars that are not reducing, like sucrose, bound glycoproteins, and glycolipids, require hydrolysis before they can take part in product formation (Arnoldie 2001). The Maillard reaction is complex in nature and the reaction pathways that lead to end products are numerous. The non-enzymatic browning involves a condensation reaction between primary amines and reducing sugars in the presence of water. The main reactive amines found in proteins are the epsilon amino groups of lysine. Another, reactive amine, is the α-amino group at the N' terminus of the polypeptide chain (Martins et al. 2001). The condensation product of the initial reaction is an N-substituted glycosylamine, which undergoes rearrangement to form an Amadori rearrangement product whose subsequent degradation is pH dependant (Martins et al 2001). The end products that are formed from the Maillard reaction are not considered digestible and it is widely held that the products are detrimental to the nutritional value of the protein, despite the fact that some intermediates are fully available in laboratory rats (Yu et al. 2002).

2.3 Assessing dietary protein

In ruminant nutrition, it is sometimes beneficial to increase the amount of dietary protein that bypasses the rumen. Thermal processing provides the means to shift the site of protein digestion to favor the small intestine, which permits the ruminant to absorb an amino acid profile that more closely resembles that of the diet. The protein remains available for absorption in the small intestine because it must pass through the abomasum which has a much lower pH than the rumen and contains different proteolytic enzymes. In addition to this, the small intestine also has its own complement of proteolytic enzymes. The result of the shift in the site of protein digestion can be of benefit to the animals under certain production and dietary conditions (Yu et al. 2002). At present, several means are used to evaluate how heat treatment will affect the degradation behaviour of protein.

2.3.1 Bypass crude protein

The major benefit to thermal processing feeds is an increase in the availability of protein to the small intestine. One measure of an increase in nutrient availability is the ruminally undegraded protein (RUP) or BCP which represents that fraction of the crude protein in a feed which will not be fermented in the rumen. BCP is based on the first order kinetics model outlined by Orskov and McDonald (1979), where $R(t) = U + D \text{ Exp}^{-Kd(t-T0)}$ and is calculated as $\%BCP = U + D * K_p / (K_p + K_d)$ and $BCP (\%CP) = 1.11 * CP * \%BCP$ using the assumption that the passage rate (K_p) is 6%/h (Yu et al. 2000). Several papers report that for different feeds, thermal processing increases BCP (Yu et al. 2000; Broderick and Craig 1980; Stern et al. 1985). It should be noted that in increasing the

amount of bypass crude protein, a corresponding drop in microbial crude protein production would be expected and the amount of absorbable BCP must increase enough to offset this difference (Yu et al. 2002). When feeds are heated to excess, increases in BCP are the result of increases in indigestible intake protein (IIP) and are related to increases in acid detergent insoluble fibre nitrogen (ADIN) content. This protein is not available to the animal and, consequently, has been demonstrated to result in poorer performance in dairy calves (Reddy and Morill, 1993).

2.3.2 Metabolizable protein

Another assessment of changes in protein availability is metabolizable protein (MP). From the NRC (2001) model, MP is true protein that is digested post-ruminally with the resulting amino acids absorbed by the small intestine and is comprised of digestible RUP (ARUP), digestible ruminally synthesized microbial CP (AMCP), and digestible endogenous CP (AECP) and is defined by the equation MP=ARUP+AMCP+AECP (Yu 2005). As shown in the equation, if thermal processing were to decrease AMCP by a greater amount than ARUP is increased, a decrease in the total MP would be expected. Despite this concern, several experiments have demonstrated that MP will increase as the degree of processing increases in various feedstuffs (Yu 2005a; 2005b). When feedstuffs are heated to excess, the consequences for MP are obvious as the ARUP fraction and AMCP fractions will decrease because less RUP would be digestible, and as a consequence, there would be less MCP produced in the rumen.

2.3.3 The DVE/OEB system

A more recent model, known as the DVE/OEB system, has been developed to evaluate dietary protein based on the strengths of previous protein evaluation models while incorporating additional elements, including the role of energy balance in protein supply. The DVE/OEB system consists of two main parts. Each feed has a DVE value representing the protein that is absorbable in the small intestine. DVE is calculated as the sum of absorbable bypass crude protein (ABCP) and absorbable microbial crude protein (AMP), less a correction for endogenous protein losses (ENDP). The second part of the system is the OEB feed value which illustrates the balance between the potential microbial protein synthesis from ruminally degradable intake protein (N_MP) and the potential microbial protein synthesis from anaerobic fermentation (E_MP). When OEB is positive, there is a potential for N loss from the rumen due to a lack of energy available to rumen microbes, whereas if the OEB value is negative, there is a lack of N available in the rumen and microbial production may be restricted (Tamminga et al. 1994; Yu et al. 2002). The DVE/OEB system provides a measure of the protein value of feeds, including the microbial protein provided by the individual feed (Tamminga et al. 1994) The reader is directed to Tamminga et al. (1994) for a detailed explanation of the DVE/OEB system.

The values for a feedstuff using the DVE/OEB system are going to change as a consequence of thermal processing. Looking at the formulas for both DVE and OEB, ABCP and BCP are present, these values are changed upon thermal processing. The question then becomes, what is the overall effect of thermal processing on the DVE/OEB value of a feed? Yu et al. (2002) reviewed the effects of thermal processing on the DVE/OEB system for several different feedstuffs. Generally speaking, the DVE value

increases with increases in temperature as well as with increasing cooking time. While endogenous protein losses are relatively constant upon heating, AMP shows a slight decrease with a larger concomitant increase in ABCP, the net result being an increase in the DVE value for that feedstuff. The OEB value, on the other hand, generally decreases as temperature and cooking time are increased. BCP is again responsible for the majority of the change in the OEB value because as BCP increases, the value for N_MP decreases. E_MP on the other hand decreases with temperature and time, due to the reduction of FOM, again due to changes in BCP, and therefore contributes less and less to the lowering of the OEB value.

2.3.4 The Cornell Net Carbohydrate and Protein System

Using the Cornell net carbohydrate and protein system (CNCPS) provides us with yet another means of evaluating ruminant dietary protein and the effects that thermal processing will have on it. The CNCPS uses *in vitro* data to characterize protein. Sniffen et al. (1992) describes in detail the classification scheme that follows. In the CNCPS, dietary protein is divided into three categories or sub-fractions, each as a percentage of CP. Fraction PA represents non-protein nitrogen (NPN) and consists of ammonia, peptides and amino acids. Fraction PC represents protein unavailable to the animal and is determined by the amount of protein insoluble in acid detergent. Fraction PC consists of protein associated with lignin, tannins, and the protein tied up in Maillard products. Fraction PB represents true protein and is further subdivided into sub fractions to permit the estimation of ruminal degradation rates. Fraction PB1 is made up of buffer soluble CP which is rapidly degraded in the rumen. Fraction PB2 represents that protein which is insoluble in buffer but is not bound to NDF. Fraction PB2 undergoes some degree of

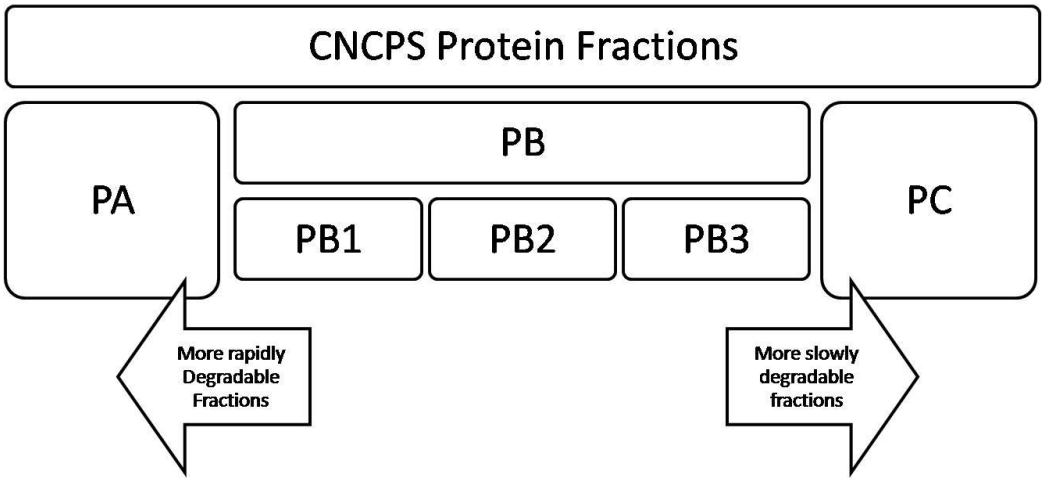

Figure 2.3. Schematic representation of protein sub-fractions in CNCPS system.

ruminal fermentation while the remainder, depending on the rates of degradation and passage, escapes to the small intestine. The final fraction, PB3, represents that protein associated with cell walls and is insoluble in neutral detergent, yet soluble in acid detergent solution. Due to the association with the cell wall, fraction PB3 ends up being slowly degraded in the rumen and as such, much of it escapes ruminal degradation.

The effects of thermal processing on the protein sub-fractions are typified by shifts in the fractions, where the rapidly degradable fractions are decreased while the more slowly degradable fractions increase. This would be expected if more protein bypassing the rumen was anticipated. In a study conducted by Yu (2005c) which compared raw and roasted flaxseed, this is exactly what was observed, fractions PA and PB1 decreased in proportion while fractions PB2, PB3 and PC increased in proportion. In a study by Shannak et al. (2000) using soybeans, dry heating induced a similar decrease in protein fractions PA and PB1, while showing an increase in the PB2 and PB3 fractions and no change in the PC fraction. In contrast, in the same study using moist heating, the PA protein fraction increased while the PB1 decreased markedly. PB2 and PB3 meanwhile increased and PC showed a slight decline. It should be concluded from this that although a general shift of fractions to the more slowly degradable ones upon heating can be expected, the changes in the fraction profile depend on the feedstuff under investigation as well as the method of applying heat. If excessive heating were to be applied and caused protein damage, major changes in the PC fraction would be expected. The PC fraction represents ADIN, and is considered a marker for heat damage and the Maillard reaction (Van Soest and Mason, 1991).

2.4 Synchrotron-based Fourier transform infrared microspectroscopy as a novel tool of feed evaluation

2.4.1 Infrared spectroscopy

Infrared spectroscopy measures the frequencies of IR light absorbed by a sample. It is through this absorption of energy that the constituent molecules in a sample of interest are identified. The commonly used device to make such measurements is a FTIR spectrometer. The FTIR spectrometer uses an interferometer which splits the incoming light into two, with one path remaining a constant distance and the other path bouncing off a moving mirror. As a result, the light travels a varying distance before it is finally recombined, run through the sample and then onto the detector (Wade 2003). The light recombines to form an interferogram containing all IR wavelengths simultaneously (Wade 2003). Initially, the interferogram is said to be in the time domain with the signal corresponding to the energy seen by the detector as the mirrors move. Fourier transform, a computer algorithm, is then applied to show the strength of absorption as a function of frequency. In other words, it is expressed in the frequency domain (Wade 2003).

Infrared or vibrational spectroscopy allows an investigator to identify which molecular bonds are present in a sample. It does so through the IR induced excitation of the bonds at wavelengths of IR light characteristic of the bond of interest. The IR frequency required to cause excitation of a molecular bond is dependent on the masses of the atoms involved in the bond and the strength of that bond or, more accurately, the stronger the bond and the lighter the atoms involved, the greater the energy required to induce bond excitation (Moore and Dalrymple, 1976). To illustrate consider the following. Triple bonds will

vibrate at a higher frequency than double bonds, and double bonds at a higher frequency than single bonds (Wade 2003). The two types of vibrations/excitations of importance in identifying organic compounds are stretching and bending, and since bending motions are easier to induce than stretching motions, they absorb light of lower frequencies or energy (Moore and Dalrymple, 1976). In addition, the bond may be stabilized or destabilized somewhat by interactions with the environment. This is demonstrated by watching the shift of the carbonyl band in polar and non-polar solvents. When surrounded by a polar environment, the hydrogen bonding that takes place serves to stabilize the bond, thus requiring IR light at a higher frequency (more energetic) to induce vibration. Other interactions include electrostatic and dispersion interactions, which may also influence vibration (Novikov et al. 1998). Not all molecules are able to be excited by IR light of any frequency. IR active bonds have a dipole moment, meaning they have some degree of charge separation between the atoms that make up the bond. This distribution of charge in the molecule is required to interact with the electric field of the IR electromagnetic radiation (Wade 2003). When the electric field is in the same direction as the dipole moment, the bond is compressed. When the electric field is changed to the opposite direction, the bond is stretched. When this stretching and compressing occurs at a frequency that matches the natural vibration state of a molecular bond, energy may be absorbed (Wade 2003). The sensitivity of IR spectroscopy to the small interactions involving the bond of interest makes it a measurement not only of that bond, but its interactions with the surrounding environment.

2.4.2 Development of synchrotron-based Fourier transform infrared microspectroscopy (S-FTIR)

Fourier Transform Infrared (FTIR) microspectroscopy is the result of the combination of microscopy with infrared (IR) spectroscopy, and enables researchers to do comparisons between the histological structures in a tissue sample and the corresponding spectroscopic chemical information (Wetzel and LeVine, 2001). These comparisons can be conducted because the microscope permits us to focus the incidental light in the experiment onto small enough regions of a sample and to isolate the histological structures in the sample. Initially, FTIR microspectroscopy was conducted on a bench top apparatus using a thermal (globar) device to generate IR light, which would then be directed through a microscope, then through the sample and finally onto the detector. This thermal source of IR light had limitations in its application, mainly due to its low brightness and the diffraction effects that occur when the aperture is reduced to sizes that correspond with the wavelength of the incoming light. The diffraction of the IR light further reduces the amount of light that can reach the detector, and consequently reduces the signal to noise ratio (S:N), in addition to scattering the light beyond the area defined by the masks (Wetzel and LeVine, 2001). This, of course, would limit the spatial resolution of the experiment as well as increase the time required to collect data. Eventual improvements in instrumentation, both IR optically efficient microscopes and spectrometers, would eventually make the light source the limiting factor in these experiments (Wetzel and Levine, 2001).

Eventually, a superior light source, the synchrotron, was applied to IR microspectroscopic experiments that would drastically improve both their spectral and

spatial resolution. This new technique is called synchrotron-based FTIR (S-FTIR). The reasons for improved spectral and spatial resolution are the intensity of the light, which is 100-1000 fold greater, the lack of noise found in thermal sources, which makes it more stable, and it is relatively non-divergent, which allows it to be highly focused with less energy lost (Wetzel and LeVine, 2001). The first two characteristics are related to increasing the S:N which generates better spectra with fewer scans. The third characteristic is related to spatial resolution (Wetzel and LeVine, 2001). The consequence of this was the ability to generate maps of the structural-chemical makeup of tissues *in situ* with each pixel representing an area that was smaller than the cells that comprised the tissue (6 µm X 6 µm) and revealed the chemical heterogeneity of the samples at those dimensions (Wetzel et al. 1998).

2.4.3 Important IR bands in feed research

Many different molecular bonds make up the biological components of a feed. Some bonds, in this context, are considered characteristic of specific biological components. The peptide bond is characteristic of protein and depicted in Figure 2.4. It is considered to be a very stable, rigid and planar bond. In the IR spectrum, two bands arise from the peptide bond in protein. These are the amide I and amide II bands. The amide I band arises from C=O stretching vibrations (80%) in addition to some contribution from C-N vibrations, with its peak in the region of ca. 1650 cm^{-1} in the IR spectrum (Jackson and Mantsch, 1995; 2000; Miller 2002; Marinkovic et al. 2002; Marinkovic and Chance, 2005; Yu 2004). The amide II band peak is found around 1550 cm^{-1} and arises from N-H bending vibrations (60%) as well as C–N stretching vibrations (40%) (Jackson and Mantsch, 1995; 2000; Yu 2004; Marinkovic and Chance, 2005). To look at changes or

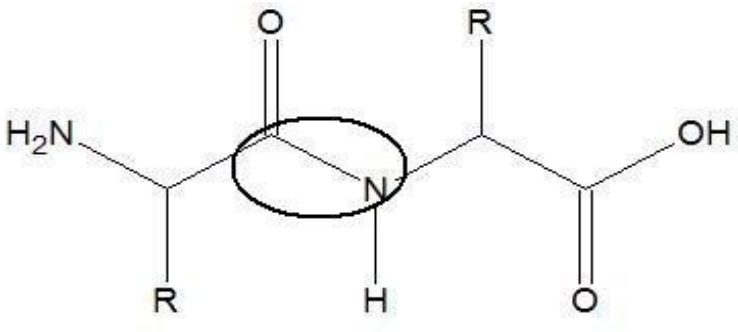

Figure 2.4. Representation of the peptide bond (*circled*) between two amino acids. R represents one of the amino acid side chains.

differences in protein structure due to processing of a feed sample, these bands would be expected to serve as an appropriate probe indicating changes to molecular structure. The amide II band, however, is considered less useful in evaluating protein secondary structure due to the complex vibrations involving many functional groups that comprise it (Haris and Chapman, 1992, Jackson and Mantsch, 1995; 2000, Marinkovic and Chance, 2005).

Lipids, as both free fatty acids and triglycerides (Figure 2.6), are also composed of characteristic functional groups (C=O ester, CH_2, CH_3) which lead to bands in the IR spectrum that are characteristic of lipids. The C=O ester band is found near 1738 cm^{-1},while the two other functional groups generate peaks near 1470 cm^{-1} (CH bending), 2961 cm^{-1} (CH_3 asymmetric stretching), 2925 cm^{-1} (CH_2 asymmetric stretching), ca. 2871 cm^{-1} (CH_3 symmetric stretching), and ca. 2853 cm^{-1} (CH_2 symmetric stretching) (Wetzel and LeVine 1999; Miller 2002; Jackson and Mantsch 2002; Yu 2004). To investigate changes in lipid structure or composition these bands could serve as appropriate probes in a manner similar to how the amide I band would be used for protein. Figure 2.6, illustrates one difference in lipid structure with implications for its spectra.
Carbohydrates (CHO) are composed of a variety of bonds between carbon (C), hydrogen (H) and oxygen (O). The resulting IR absorption bands are quite complex making their interpretation complex as well. CHO generates strong bands in the ca. 1550-800 cm^{-1} spectral region, in particular around 1185-800 cm^{-1} (Wetzel et al. 1998; Yu 2004). There are some bands (ca. 1420 cm^{-1}, 1370 cm^{-1}, 1335 cm^{-1}), however, that can be used to indicate the presence of structural (cellulose) CHO in a sample (Wetzel et al. 1998). The band near 1420 cm^{-1} has been more specifically associated with β-glucan (Wetzel et al.

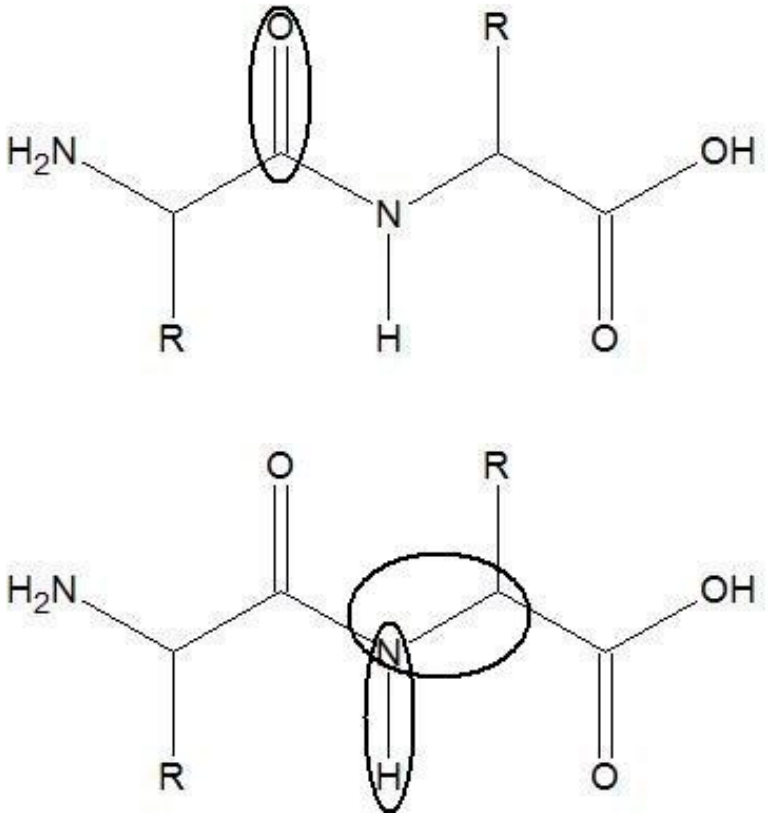

Figure 2.5. Major bonds responsible (*circled*) for the amide I band (*top*) and amide II band (*bottom*) in the mid-IR spectrum.

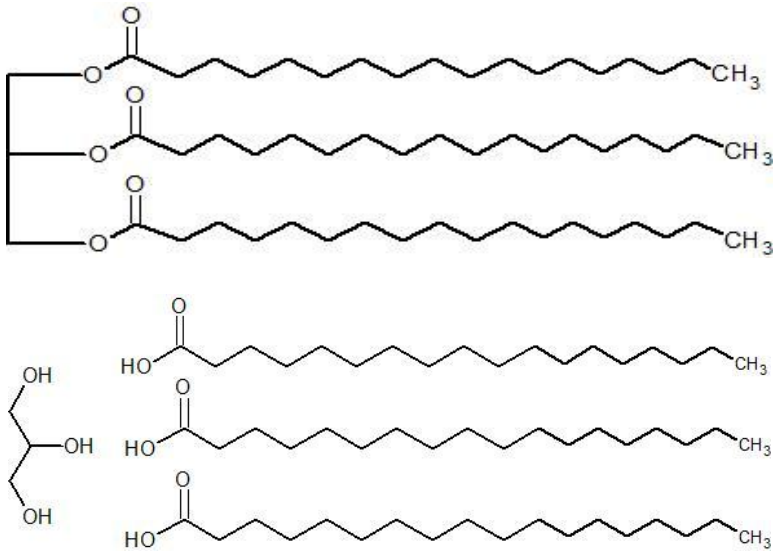

Figure 2.6. Molecular differences in lipid structures. *(bottom)* Free fatty acids depicted with the 3 C glycerol. *(top)* Triglyceride from the same fatty acids. Fatty acids chains also vary themselves in composition in terms of chain length, saturation, and double bond locations. (Voet and Voet, 1995a)

1998). Other bands considered to be indicative of different CHOs include ca. 1025 cm^{-1} (non-structural CHO starch), ca. 1510 cm^{-1} (aromatic lignin), ca. 1246 cm^{-1} (cellulosic compounds), ca. 1100 cm^{-1} (cellulose) (Wetzel et al. 1998; Yu 2004). Figure 2.7 illustrates how the different bonds lead to a polymer of the same subunits with a very different structure.

2.4.4 Assessing feeds with IR spectroscopy

It has been suggested that the protein matrix that surrounds each starch granule is important with respect to how quickly the starch in various cereal grains will degrade (McAllister et al. 1993). In a study conducted by Yu et al. (2004a), two different barley varieties were selected based on similarities in traditional chemical values (starch, CP, etc.), but also on differences in their digestion characteristics. Harrington, a quickly and extensively degraded malting barley, and Valier, a slowly and limited degrading feed type barley, were selected to probe whether differences in the starch-protein matrix were responsible for their differences in digestive behaviour. The starch-protein matrix was assessed by the starch to protein band intensity ratio between the two varieties. The study showed that the starch to protein band intensity ratio was the same for both Harrington and Valier. Harrington, however, showed a greater range in that ratio, and it was concluded that this was a result of greater heterogeneity of the starch-protein matrix in the endosperm. It was concluded that the association of starch with protein, as defined by the band intensity ratios, was not the sole cause of differences in degradation behaviour. It would appear. however, that heterogeneity of the starch-protein matrix itself may have

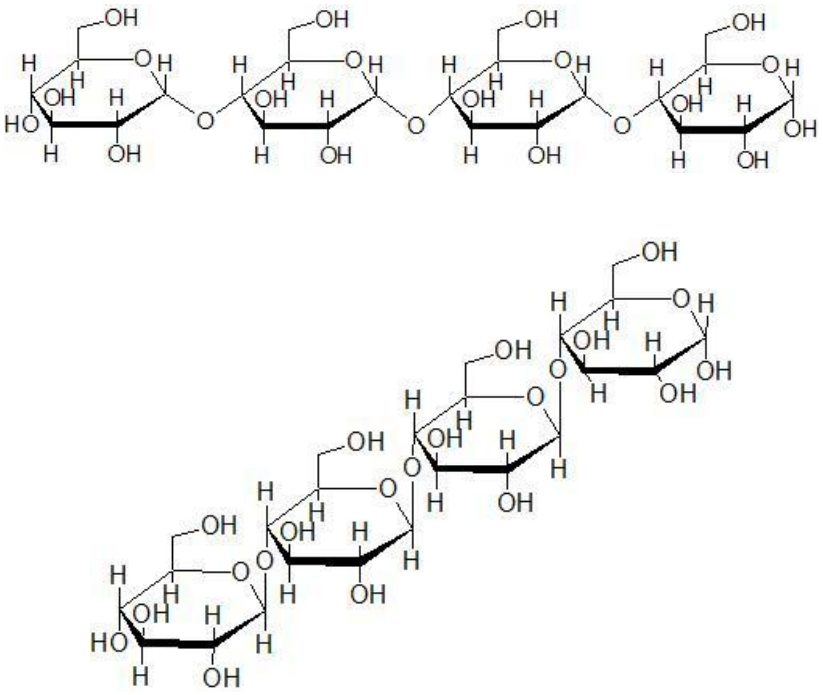

Figure 2.7. Difference in the molecular structure of common bonds found in starch (α-1-4 linkage) *(Top)* and those found in cellulose (β-1-4 linkage) *(Bottom)*. The different bonds have effects on the polymer structure. (Voet and Voet, 1995b)

some potential as an assessment of sub-cellular structure as it influences degradation behaviour.

S-FTIR can be used to characterize solely the protein in the endosperm of a feed by focusing specifically on those bands that result from the peptide bonds of protein. These are known as the amide I and amide II bands, as previously mentioned. Prior to the Yu et al. (2004a) study, it had been shown that the amide I to amide II ratio decreased in scrapie-infected tissue (Miller and Dumas, 2006; Wang et al. 2005). Scrapie is known to result in secondary structure changes in prion proteins from α-helices to β-sheets. Therefore, a difference in this ratio is indicative of some difference in protein structure. It is possible that protein structure plays a role in degradability, so this ratio may permit the evaluation of protein structure in a manner which could be linked to degradability. Yu et al. (2004a) results showed in fact, that there was a difference in both band intensities between two varieties of barley (Harrington and Valier) with different rumen degradation rates. The ratio was slightly smaller for Harrington, and it was concluded that there was some structural difference between the two varieties that led to the results. There is little published data, however, on the relationship between these values and nutrition.

Because the amide I and amide II bands are predominantly from protein, they exhibit shifts in their location in the spectrum depending on the secondary structure in which the peptide bonds are located. The frequency shift permits them to be used to determine the ratios of secondary structure that make up the protein in a feed (Wetzel et al. 2003). The amino acids in a protein are found in α-helix, β-sheet conformations, but also in β-turn and random coil conformations. The former conformations are periodic while the latter are aperiodic. Each of these secondary structures provides a different environment for the

amide I bonds, resulting in shifts in the location of the peak for the amide I band in the spectrum (Marinkovic et al. 2002; Marinkovic and Chance, 2006). This means that the bands are actually made up of multiple elements blurred together, where the contribution of each is concentration dependant and requires mathematical treatment to isolate the contribution of each element (Marinkovic and Chance, 2006; Marinkovic et al. 2002). To separate the individual peaks, the spectrum first undergoes Fourier self-deconvolution (FSD). Then, to quantify the area of each of the peaks in the FSD spectrum, multi-peak fitting software is used, thus providing us with relative values for the amount of each secondary structure (Yu 2006b).

It is important to note that concentration values for protein secondary structures, obtained from mid-IR spectroscopic data, are only relative and not exact determinations (Yu 2006a). Important factors in IR spectroscopy are responsible for this fact, and include different molar absorptivities, the number of component bands, component band shapes and noise (Yu 2006a). In addition to this, researchers do similar analyses but select different-sized spectral regions, different baselines and different mathematical manipulations of the spectra (Yu 2006a). The relative values that are derived are still useful, however, for comparing treatments, but there are several considerations one needs to be aware of regarding these determinations.

Due to the actual physical differences in these secondary structures, there may be some difference in rumen degradability of the protein in a feed. Examining the protein secondary structures of feather, a poorly degraded protein source, showed a much lower α-helix to β-sheet ratio than that found in more easily degraded protein sources (Yu et al. 2004b). Subsequently, the study between the two barley varieties showed no difference in

the α-helix:β-sheet ratio. There were differences when the α-helix and β-sheet were compared to other elements of secondary structure, leading to the conclusion that there were, in fact, detectable structural differences between the two cultivars (Yu et al. 2008). In a separate study, Yu et al. (2005) used two types of flaxseed (golden and brown), both raw and roasted, to determine if cooking would change the α-helix:β-sheet ratio. The results showed that roasting did reduce the α-helix:β-sheet ratio of the golden flaxseed, but not the brown flaxseed, indicating that different feeds will exhibit different sensitivities to changes in protein secondary structure upon heating. It was further shown (Yu et al. 2005) that the changes in protein secondary structure were accompanied by changes in protein degradability in flaxseed. The picture that remains is that protein secondary structure differences are indicative of differences or changes in protein degradability, but caution should be used when comparing different feeds as they may respond differently to treatment.

2.4.5 Multivariate statistical methods for spectral comparison

The previously mentioned measurements focus on direct measurements of specific characteristics of the IR spectrum of a feed that are compared using univariate methods of analysis. Multivariate analysis permits the researcher to use all or large portions of the spectral data at once to do comparisons, regardless of what the bands in the spectra specifically represent (Yu 2006b). Multivariate methods used in spectroscopic research include principal component analysis (PCA) and hierarchical cluster analysis (CLA). The purpose of these procedures is to group the spectra according to their similarity to one another, but they use different methods to do comparisons.

CLA calculates a distance matrix which contains information about the similarity of the spectra. Then it groups the most similar spectra based on the distance matrix to create a cluster which is followed by the recalculating the distances of the remaining spectra to the new cluster (Yu 2006b). CLA, when used in feed research, is able to distinguish between different varieties of the same feedstuffs in addition to different treatments of the same variety (Yu 2005d).

PCA, meanwhile, constitutes a statistical data reduction method that transforms the original variables in the data set into a new and uncorrelated set of values called principal components that still account for the original variance in the data (Yu 2005d). Each spectrum is assigned a score (eigenvector) defining its relationship to each principal component. The eigenvector is then used to create 2-D or 3-D scatter plots, on which proximity of spectra to one another indicates similarity between the spectra. For a more detailed explanation of PCA, the reader is directed to Dunteman (1989). As with CLA, PCA has also been shown to be able to distinguish between different varieties of the same feedstuffs in addition to different treatments of the same variety (Yu 2005d). While PCA and CLA used in this manner do not provide structural information per se, they imply molecular differences or similarities in the mid-IR spectroscopy data that is used.

2.4.6 The need for S-FTIR use in feed research

S-FTIR ultimately provides us with information relevant to the nutritional characteristics of a particular feed because these characteristics are influenced not only by total composition of a feed, but also by morphological characteristics and biological component matrices (Yu 2006b). The information about the latter two is typically lost

during most chemical analyses and this loss of information, or the inability to account for it, is believed to be accountable for the failure of models such as the NRC 2001 model to fully predict nutritive or energy values for a given feed (Yu et al. 2004a). In addition, S-FTIR is a rapid and non-destructive technique so the sample remains intact and unaltered by the experimental process, allowing further assaying of the sample if desired. The problems for the investigator that remain are connecting the elements of structure that are revealed by S-FTIR (or changes to those elements) to their influence on digestive behaviour and determining if the sample, from a sub-cellular area, is representative of the feed as a whole. Another concern is that the sample preparation process, prior to spectroscopic analysis, will itself affect the spectrum from a given sample so caution must be exercised to ensure the samples are treated identically.

3. OVERVIEW OF RESEARCH TRIALS

The two studies that follow are complementary; both provide specific information about the protein chemistry of a feed. There exist many established methods and models that use specific chemical analyses to create a picture of how an ingested feed will interact with the animal. Mid-IR spectroscopic methods, capable of providing an alternate kind of chemical information with links to many levels of physical structure and chemistry, are well established. Physical structure, from histology down to the macromolecular level, plays a large role in how the digestive system will be able to interact with a feed. How the digestive system interacts with feed as it passes through is critical to a feed's nutritional quality as a result. Spectroscopic chemical information provides a measure of all the molecular bonds in a sample, including interactions of those bonds with their immediate environment. The ability to link both types of chemical information to one another could prove to be invaluable to the accuracy of the feed models, but may also allow us to develop a more rapid method of evaluating a feed accurately. Including both types of information into current models can only serve to improve the models as well as our understanding of the digestive process and influencing factors. This is the goal of the companion studies that follow.

The first study, which used established models based on *in vitro* and *in situ* chemical and nutritional analyses, was to demonstrate differences in protein degradability, degraded protein balance, RUP and CNCPS protein sub-fractions that result in flaxseed as a consequence of thermal treatment by autoclaving without physical disruption. Using this method of heating, chemical and structural changes that occurred over time were evaluated. In this study, chemical changes were evaluated based on existing models and

were then used subsequently for comparison to mid-IR spectroscopic data from the second study. These traditional methods of evaluating feed cannot account for certain structural elements that may be involved in nutrient degradability and have been consequently demonstrated to lack the ability to predict with great precision differences in degradability and energy between similar yet known to be different feeds, such as malting vs. feed type barleys (Yu et al. 2008). The inability to take these elements or structures and factor them into current models is a result of the common methods used to characterize a feed. These often include harsh acid and detergent treatments, solubilizing, derivitization and grinding. These treatments ultimately are not sensitive to all the information required to adequately describe a feed so that reliable predictions can be made about its nutritive value (Yu 2004).

The S-FTIR study was an attempt to bridge the structural information gap that exists in current models. The infrared analysis of flaxseed from study 1 sought to determine how sensitive S-FTIR was to thermally induced changes in flaxseed and whether those changes, when quantified, could be used as measures of protein degradation behaviour. S-FTIR can be used to ascertain different levels of structure in a feed that may be of use in improving current models. As S-FTIR allows the investigator to non-destructively probe sub-cellular regions of a sample, it can be used to describe a feed chemically at that level, which reveals things such as heterogeneity and micro-localization of nutrients of a particular feed component, whether it is protein or starch (Wetzel et al. 1998). S-FTIR also provides information about the structure of the nutrients themselves, since differences in biological polymer conformations are readily identifiable from the spectra, as shown through protein secondary structure analysis (Yu 2006b, Bonwell et al. 2008).

Both these levels of feed structure are considered to play some role in protein degradability (McAllister et al. 1993, Yu et al. 2005).

The hypothesis behind these studies is that chemical and structural changes which are induced thermally are demonstrable by mid-IR spectroscopy. These changes can be correlated to differences in degradability as shown by established chemically-based methods. If a correlation can be established, then protein degradability may have specific signatures in the mid-IR spectrum which could be used to improve current models of estimating nutrient degradability or provide some alternate measure of degradability.

4. *IN VITRO*/*IN SITU* NUTRITIONAL CHARACTERIZATION AND PREDICTED NUTRIENT SUPPLY OF FLAXSEED PROTEIN AS MODIFIED BY AUTOCLAVING

4.1 Introduction

Recently, flaxseed has garnered interest as a functional food source in human nutrition. This is due, in large part, to the high content of ALA in flaxseed, but it is also a good source of high quality protein and lignan, all of which may benefit the health of the consumer (Oomah 2001). Flaxseed is also generating interest as a functional feed source for ruminant production systems. The varied interest in flaxseed is due to the same components, however, the benefits are multifaceted. Firstly, ruminants fed flaxseed show improvements in reproductive health indicators. Petit and Twagiramungu (2006) found a decrease in embryo mortality, and consequently improved the establishment of pregnancy when feeding flaxseed compared to Megalac and extruded soybeans. Ambrose et al. (2006) found a similar decrease in pregnancy losses when feeding flaxseed compared to sunflower seed. Secondly, the fatty acid profile of both milk and meat products changes upon feeding flaxseed, as demonstrated by Petit (2002) and Scollan et al. (2001a). The changes in fatty acid composition are typified by a shift in the n-6:n-3 ratio which, in the first case, is a benefit to the producer, whereas in the second case the benefit is mainly to the consumer of ruminant milk and meat products. Depending on how the producer can market his milk or meat, it may create a niche market for a value-added product with the potential for increased returns.

Often, in highly productive animals there is an improvement in productivity when a greater amount of the dietary amino acids can reach the small intestine for absorption. This is commonly achieved for oilseeds and oilseed meals by heating (McKinnon et al. 1995; Mustafa et al. 2003a; 2003b). The effects that thermal processing will have on degradability depend on the method used to apply heat, the presence of moisture, temperature and time. Petit et al. (2002) demonstrated that a moist heating environment was more efficient than dry heat at increasing RUP at 100°C but at 120°C there was much less difference in ruminally undegraded protein (RUP) between the heating methods. This indicates the complexity of the effects heating has on a feed. The overall seed structure was not changed during heating yet somehow the presence of moisture is a critical factor at certain temperatures, perhaps acting as a catalyst for the reactions that cause changes in protein degradability. When extrusion is used as a method of heat application, the consequences to RUP can be the opposite. Mustafa et al. (2003a) demonstrated this while extruding at a temperature of 155°C and with a 43 sec residence time. The conclusion from this study was that the conditions of extrusion used were ineffective at increasing the post ruminal supply of dietary amino acids and that the abrogation of the oil/protein matrix could explain this result. To date, little information is available about flaxseed and few studies examine how variations in the same heating process would influence protein chemistry and degradability.

Given the potential value of adding flaxseed to the ruminant diet, this study sought to add to the body of information on flaxseed as a feed ingredient. At present, few studies cover the protein chemistry and degradability changes that occur to flaxseed upon heating. The hypothesis is that flaxseed exposed to autoclave heat treatment will experience changes to

its nutritional profile. The goal of this study is to describe the effects of autoclaving whole flaxseed in terms of protein degradability, CNCPS protein sub-fractions, *in vitro* intestinal digestibility and RUP. Secondly, the objective was to provide a basis for evaluating FTIR spectroscopic data in a companion study as a means of studying protein structure and degradability in feeds.

4.2 Materials and Methods

4.2.1 Flaxseed heating and processing

Flaxseed (*Linum usitatissimum* L. cv. Vimy) grown at Moose Jaw, Saskatchewan during the 2005 growing year was provided by Shamrock Seeds Ltd. Saskatoon, Saskatchewan. Three-kilogram samples of flaxseed were heated by autoclave (Amsco Eagle SG-3031, STERIS Corporation, Mentor, Ohio, USA) at 120°C in gravity mode for 20, 40 or 60 min. Treatments were then repeated on a separate set of 3-kg samples providing two sets of treatments, A and B. Control treatments were unheated. Samples were subsequently cooled to room temperature and then placed in the refrigerator prior to grinding. The samples were ground using a Retsch SM 2000 (Retsch Inc., Newtown, PA, USA) grinder fitted with a 2-mm screen. Samples were fed slowly into the grinder to prevent adhering during the grinding process and to ensure the flaxseed was cracked open and not "extruded" through the screen.

4.2.2 Animals and diets

Two non-lactating Holstein dairy cows fitted with flexible rumen cannula (10 cm internal diameter, Bar Diamond Inc., Parma, ID, USA) were used to determine the *in situ*

degradation kinetics and effective degradability of flaxseed. The cows were housed in an experimental station (Stone Barn) at the University of Saskatchewan during the period of study. Pens were approximately 6 by 9 metres. The cows were individually fed a totally mixed ration (15 kg/day as fed) containing 60% barley silage (35% DM), 26% dairy concentrate, 10% alfalfa hay and 4% alfalfa dehydrate according to non-lactating dairy cow requirements (NRC 2001). Concentrate consisted of barley, wheat, oats, molasses and dairy supplement pellets which were comprised of soybean meal, canola meal, wheat, dried distillers grains, corn gluten meal, molasses, barley, cobalt iodized salt, canola oil, sodium bicarbonate, Dynamate [22% Sulfur, 18% potassium, 11% magnesium (International Minerals and Chemical Corp., Mundelein, ILL, USA)] and a mineral vitamin mix (formulated to provide 45 mg manganese, 63 mg zinc, 17 mg copper, 0.5 mg selenium, 11,000 I.U. vitamin A, 1800 I.U. vitamin D_3 and 30 I.U. vitamin E per kg of dairy concentrate). The mix also contributes 0.14% magnesium, 0.48% calcium, 0.26% phosphorus, 0.23% sodium and 0.38% chloride to the total dairy concentrate (Prepared by Federated Cooperatives Ltd., Saskatoon, SK, CAN). The cows were fed half of the ration twice daily at 0800h and 1600h. Water was available *ad libitum.* The animals in this experiment were cared for according to the guidelines of the Canadian Council on Animal Care (1993).

4.2.3 Rumen incubation

Rumen degradation kinetics and effective degradability were determined using the *in situ* method. Seven-gram samples were placed into numbered bags measuring 10 cm x 20 cm made of Nitex 03-41/31 monofilament polyamide open mesh fabric (Screentec Corp., Mississauga, ON, CAN) with a pore size of 41 µm. The seams of the bags were sewn

shut and sealed with glue to prevent samples from escaping during incubation. The sample bags were placed in a polyester mesh bag (45 cm x 45 cm with a 90 cm length of rope to anchor it to the cannula) weighed down with a plastic bottle (250 mL) filled with gravel to keep the samples in the liquid strata of the rumen. Bags were added according to the 'gradual addition/all out' schedule and were incubated for 72, 48, 24, 12, 8, 4, 2 or 0 h. The number of bags incubated from each sample was increased based on incubation time to ensure an adequate amount of residue would remain for analysis. Estimates for the amount of sample required for incubation were obtained using data from Mustafa et al. (2003a). All treatments for every incubation period were incubated in duplicate (2 runs) in two non-lactating dairy cows. All bags were randomly assigned to each of the two cows at the time of incubation. A maximum of 35 bags were incubating in the rumen at any one time. After incubation bags were rinsed with cold water then washed in groups of ten with 2 litres of water 5 times so the water from the last rinse cycle would run clear. Washed bags were then dried at 55°C in a forced air oven for 48 h. After drying the bags were stored in plastic bags in a refrigerator.

4.2.4 Chemical analysis

Dried samples were pooled and ground through a 1 mm screen using a Retsch ZM 100 (Retsch Inc., Newtown, PA, USA). Samples were then analyzed for dry matter (AOAC method 930.15), ash (AOAC method 942.05), ether extract (AOAC method 920.39), crude protein (AOAC method 984.13) (2400 kjeltech analyzer unit, Foss Tecator, Edenprairie, MN, USA), ADIN (Licitra et al 1995), NDIN (Licitra et al 1995), NPN (Roe et al. 1990) and starch (AOAC method 996.11, using Megazyme Total Starch assay). ADF, NDF and ADL were analyzed by the Ankom filter bag method (ANKOM A200

Filter Bag technique, Ankom Technology, Fairport, NY, USA) with a modified fat extraction procedure. The procedure was modified to include an initial 2 h ether extraction along with the standard Ankom acetone fat extraction protocol, to prevent the high fat content of the flaxseed samples from giving erroneously high values for the fibre measurements.

4.2.5 Fractionation of protein fractions according to the Cornell Net Carbohydrate and Protein system

The CP fractions were partitioned according to the Cornell Net Carbohydrate and protein system (CNCPS) (Sniffen et al. 1992). There are five CP fractions as determined by the CNCPS (PA, PB1, PB2, PB3, PC) and they are calculated as follows:

PA (%CP) = NPN (%SCP) * 0.01 * SCP (%CP)

PB1 (%CP) = SCP (%CP) – PA (%CP)

PB2 (%CP) = 100 – PA (%CP) – PB1 (%CP) – PB3 (%CP) – PC (%CP)

PB3 (%CP) = NDIP (%CP) – ADIP (%CP)

PC (%CP) = ADIP (%CP)

Where, NPN (%SCP) is non protein nitrogen as a percentage of soluble crude protein; SCP (%CP) is soluble crude protein as a percentage of crude protein; NDIP (%CP) is neutral detergent insoluble protein as a percentage of crude protein; and ADIP (%CP) is acid detergent insoluble protein as a percentage of crude protein.

4.2.6 Rumen degradation characteristics

Rumen degradation kinetics of the raw and autoclaved flaxseed were determined for DM and CP. Degradation kinetics were determined using a first order rumen degradation equation modified to include lag time outlined by Orskov and McDonald (1979) as shown below.

$$Y = S + D\,(1 - Exp^{-Kd(t - T0)})$$

Where: Y represents the amount of sample that has disappeared at time t; D represents the potentially degradable fraction (%); S represents the soluble fraction (%) of the residue; K_d represents the degradation rate (%/h.); and T0 represents the lag time (h) for the sample to begin degrading. The model was fitted to the data using PROC NLIN of SAS 9.1(SAS Institute Inc., Cary, NC, USA). via the Gauss-Newton method which is an iterative non-linear regression protocol.

The effectively degraded (ED) and bypass (B) fractions for DM and CP were also considered and calculated according to formulas below as outlined in NRC (2001).

$$ED = S + D\,(K_d / K_d + K_p)$$

$$B = U + D\,(K_p / K_d + K_p)$$

Where, K_p represents the rate of passage and is assumed to be 6%/h.

4.2.7 Metabolizable protein prediction with the NRC (2001) model

Metabolizable protein (MP) refers to true protein that is digested postruminally. According to NRC (2001), it is composed of three sources of protein, more specifically the absorbable fractions of the three protein sources. MP was calculated as follows:

$$\text{MP (g/kg DM)} = \text{AMCP (g/kg DM)} + \text{ARUP (g/kg DM)} + \text{AECP (g/kg DM)}$$

Where, AMCP refers to absorbable microbial crude protein; ARUP refers to absorbable ruminally undegraded feed protein in the intestines; and AECP refers to the absorbable endogenous crude protein.

Calculation of the absorbable fractions requires the consideration of each protein source and its respective degradability. With AMCP, for example, MCP is considered only 80% true protein with the remainder being nucleic acids. The true protein fraction of MCP is considered to be 80% digestible. As a result, AMCP was calculated as follows:

$$\text{AMCP (g/kg DM)} = \text{MCP (g/kg DM)} * 0.8 * 0.8$$

The yield of MCP is assumed to be 130 g MCP/kg TDN (discounted). The requirement for MCP yield is RDP. Specifically, RDP must be greater than 1.18 * MCP yield. Under these conditions, MCP is calculated as:

$$\text{MCP (g/kg DM)} = 0.130 * \text{TDN (g/kg DM) (discounted)}$$

When RDP is less than 1.18 * MCP (TDN predicted yield), MCP is calculated as follows:

$$\text{MCP (g/kg DM)} = 0.85 * \text{RDP (g/kg DM)}$$

The digestibility of RUP (dRUP) in this study was determined specifically for each flaxseed treatment using the three-step *in vitro* procedure as described by Calsamiglia and Stern (1995).

Therefore, ARUP is calculated as:

$$\text{ARUP (g/kg DM)} = \text{RUP (g/kg DM)} * \text{dRUP}$$

ECP contributions of a feed were estimated according to NRC (2001) as follows:

$$\text{ECP (g/kg DM)} = 1.9 * \text{DM} * 6.25$$

The ECP fraction is considered to be only 50% true protein and as MCP is considered to be 80% digestible, AECP was calculated as:

$$\text{AECP (g/kg DM)} = \text{ECP (g/kg DM)} * 0.5 * 0.8$$

The degraded protein balance (DPB), outlined by Yu et al. (2003), illustrates the difference between the RDP of a feed and the RDP required for MCP synthesis. The (DPB) was calculated as:

$$\text{DPB (g/kg DM)} = \text{RDP (g/kg DM)} - 1.18 * \text{MCP (g/kg DM)}$$

4.2.8 Modeling nutrient supply with the truly digested protein (DVE)/ degraded protein balance (OEB) system

The DVE/OEB system is outlined by Tamminga et al. (1994). It constitutes a two part system where each feed has a DVE and an OEB value. The DVE value is comprised of digestible feed protein, microbial protein and an endogenous protein loss correction. The OEB value, or degraded protein balance, reflects the difference between potential

microbial protein synthesis based on degraded feed protein and the potential microbial protein synthesis based on the energy available for microbial fermentation in the rumen.

DVE was calculated as:

$$\text{DVE (g/kg DM)} = \text{AMCP (g/kg DM)} + \text{ARUP (g/kg DM)} - \text{ENDP (g/kg DM)}$$

Where, AMCP is the absorbable fraction of microbial crude protein; ARUP is the absorbable fraction of ruminally undegraded feed protein; and ENDP is a correction factor for endogenous protein lost during the digestion process. Due to differences in the DVE/OEB system and NRC (2001), these values, while representing similar protein fractions, are calculated differently.

MCP production in the DVE/OEB system is based on fermentable organic matter (FOM), calculated as:

$$\text{FOM (g/kg DM)} = \text{DOM (g/kg DM)} - \text{EE (g/kg DM)} - \text{RUP (g/kg DM)} - \text{RUst (g/kg DM)} - (0.50) * \text{FP (g/kg DM)}$$

Where, DOM represented the organic matter that had disappeared after 72 h of rumen incubation; EE represented the ether extract; RUP represented ruminally undegraded feed protein; RUst represented ruminally undegraded starch; and FP represented the fermentation products of ensiled feeds.

AMCP then was calculated as follows:

$$\text{AMCP (g/kg DM)} = \text{FOM (g/kg DM)} * 0.150 * 0.75 * 0.85$$

Where, FOM in the DVE model is assumed to produce 150 g MCP/kg FOM, of which 75% is considered to be true protein that has an assumed digestibility of 85%.

ARUP in the DVE system was calculated as:

$$\text{ARUP (g/kg DM)} = \text{CP (g/kg DM)} * (1.11 * \text{RUP (\%CP)} / 100) * (\text{dRUP\%} / 100)$$

ENDP is a correction factor for the N that is lost as a consequence of the various digestive processes and is linked to undigested DM (UDM). ENDP also includes a correction factor due to losses of metabolic protein and the efficiency of resynthesis. ENDP is calculated as:

$$\text{ENDP (g/kg DM)} = 0.075 * \text{UDM (g/kg DM)}$$

UDM is composed of indigestible organic matter and indigestible inorganic matter and is calculated on a g/kg basis, hence:

$$\text{UDM (g/kg DM)} = 1000 - \text{DOM (g/kg DM)} - \text{VRAS (g/kg DM)}$$

Where, VRAS is digestible inorganic matter

The degradable protein balance or OEB value of a feed is the difference between the potential MCP synthesis based on RDP (MCP_{RDP}) and the potential MCP synthesis based on energy extracted from anaerobic fermentation (MCP_{FOM}). Therefore,

$$\text{OEB (g/kg DM)} = MCP_{RDP} \text{ (g/kg DM)} - MCP_{FOM} \text{ (g/kg DM)}$$

Where, MCP_{RDP} is calculated as:

$$MCP_{RDP} \text{ (g/kg DM)} = \text{CP (g/kg DM)} \times [1 - (1.11 \times \text{RUP(\%CP)}/100)]$$

And MCP_{FOM} is calculated as:

$$MCP_{FOM} \text{ (g/kg DM)} = FOM \text{ (g/kg DM)} * 0.15$$

4.2.9 Determination of intestinal protein digestion

In vitro estimates of intestinal digestibility were determined according to the protocol for ruminants outlined in Calsamiglia and Stern (1995). The incubation schedule did not include a 16 h incubation period, so the residues used for analysis were incubated for 12 h and not 16h as called for.

4.2.10 Statistical analysis

Statistical analyses were performed using the MIXED procedure of SAS (version 9.1) (SAS Institute Inc., Cary, NC, USA) using a completely randomized experimental design (CRD) with the following model

$$Y = \mu + T + \boldsymbol{e}$$

Where, μ is the mean for the variable of interest; R is the block effect using the run as a block; T is the treatment effect; and $\boldsymbol{e}$ is the random error of observation. Means were separated using the LSD method with significance declared at $P < 0.05$.

4.3 Results and Discussion

DM and EE increased ($P < 0.05$) from 90.7 to 92.8 (%DM) and 42.1 to 44.5 (%DM), respectively, upon autoclave heating, whereas ash and OM remained unchanged Table 4.1. Results for ash content were similar to those published by Gonthier et al. (2004). There was no significant difference ($P < 0.05$) between the autoclave treatments for either

Table 4.1 Chemical composition of the raw (control) compared to the autoclave treated Vimy flaxseed.

Component	Control	Autoclave Treatment (120°C)			SEM
		20 min	40 min	60 min	
Dry Matter (%)	90.7[b]	92.5[a]	92.8[a]	92.8[a]	0.07
Ether Extract (%DM)	42.1[b]	44.1[a]	44.5[a]	44.2[a]	0.34
Ash (%DM)	3.5	3.6	3.6	3.5	0.01
Organic Matter (%DM)	96.5	96.4	96.5	96.5	0.01
Crude Protein (%DM)	23.9	23.7	23.8	23.8	0.13
Soluble Crude Protein (%DM)	12.4[a]	8.5[b]	5.4[c]	4.5[c]	0.32
Non-Protein Nitrogen (%DM)	1.4[b]	1.3[b]	1.5[b]	2.0[a]	0.10
Neutral Detergent Insoluble Protein (%DM)	3.8[b]	4.1[b]	4.4[b]	5.7[a]	0.13
Acid Detergent Insoluble Protein (%DM)	0.5[c]	0.6[bc]	0.7[ab]	0.8[a]	0.04

[a, b, c,] Means with the same superscripts in the same row are not significantly different ($P < 0.05$). Means separated using the LSD method

SEM = Standard error of mean

DM or EE. Values for EE of the raw Vimy flaxseed samples were very similar to those reported by Oomah and Kenaschuck (1995). The slight increase in the EE fraction is interesting to note and has been observed in ground sunflower seeds treated similarly by Mustafa et al. (2003b), and also in roasted whole soybean and cottonseed by Mohamed et al. (1988). This would appear to be an artefact as somehow heating increases the amount of fat that is soluble in ether. This effect is used advantageously in the oil extraction industry, and it is held that the heating results in the rupture of the oil cells and permits the coalescing of larger droplets of oil (Booth 2004). Under autoclave or roasting conditions, with no physical disruption directly from heating, there is a reaction taking place which is affecting the physical properties of the components making up the oil bodies which results in the change in solubility. The disruption of oil bodies through the heating process is likely to occur in one of two ways: a) through a physical change such as denaturation which would remove the effect of steric hindrance exposing the inside of the oil bodies to one another allowing them to coalesce; or b) through changes to the charged surfaces of the protein responsible for the structure of the oil protein matrix (Huang 1992).

The total carbohydrate content of the raw and the treated flaxseed showed a decrease ($P < 0.05$) upon autoclaving from 30.4 to 28.1 (%DM) (Table 4.2). This difference arises from the calculation of total CHO by subtracting ash (%DM), EE (%DM) and CP (%DM) from 100. Due to the change in the EE fraction, less CHO was calculated for the autoclave treatments by this method. NDF and ADF decreased ($P < 0.05$) from 17.2 to 13.9 (%DM) and 10.2 to 7.7 (%DM), respectively, although there was no significant difference ($P < 0.05$) between the autoclave treatments. These results for ADF and NDF paralleled a

Table 4.2 Carbohydrate profiles of the raw (control) compared to the autoclave treated Vimy flaxseed.

		Autoclave Treatment (120°C)			
Component (%DM)	Control	20 min	40 min	60 min	SEM
Carbohydrate[1]	30.4[a]	28.7[b]	28.1[b]	28.5[b]	0.35
Neutral Detergent Fibre	17.2[a]	14.0[b]	13.9[b]	14.5[b]	0.31
Acid Detergent Fibre	10.2[a]	7.7[b]	7.8[b]	7.9[b]	0.31
Acid Detergent Lignin	2.2[b]	2.4[ab]	2.7[ab]	2.8[a]	0.14
Starch	0.3	0.3	0.3	0.3	0.23
Non-Structural Carbohydrate[2]	17.0	18.8	18.6	19.7	0.69
Hemi-cellulose[3]	7.1[a]	6.3[b]	6.1[b]	6.6[ab]	0.14
Cellulose[4]	7.9[a]	5.3[b]	5.2[b]	5.0[c]	0.22

[a, b, c,] Means with the same superscripts in the same row are not significantly different ($P < 0.05$). Means separated using the LSD method

SEM = Standard error of mean

[1]Carbohydrate = 100 – EE –CP – Ash

[2]Non-Structural Carbohydrate = 100 – (NDF – NDIP) – EE – CP - Ash

[3]Hemi-cellulose = NDF - ADF

[4]Cellulose = ADF – ADL

study by Mustafa et al. (2003b) where both NDF and ADF decreased initially upon autoclave heating, but with longer heating times the decrease became less prominent. The results from this study show a similar inflection in NDF and ADF values with increasing heating time; however, the values were not significantly different from one another. The NDF and ADF values from this study were similar to those reported for raw flaxseed by Gonthier et al. (2004). ADL showed a small increase ($P < 0.05$), from 2.2 to 2.8 (%DM), with autoclaving. However, the only treatment significantly different from the raw samples was the 1-h autoclave treatment. Starch composition of the flaxseed samples was negligible in all cases. No significant differences were detected for non-structural carbohydrates, although there was a numerical increase in the values. This value is determined by the difference method and also contains the artefact associated with the EE value. This would skew the results downward about 2% on a DM basis for the treatments compared to the raw samples. Hemicellulose decreased ($P<0.05$) from 7.1 to 6.1 (%DM) upon autoclaving, although no differences were detectable between the autoclave treatments. Cellulose also showed a decrease ($P < 0.05$) upon autoclaving, decreasing from 7.9 to 5.0 (%DM), with the 1-h autoclave treatment being statistically different ($P < 0.05$) from the other treatments. Nevertheless, it was numerically similar to the shorter treatment times.

No significant changes were observed in the CP measurements upon autoclaving (Table 4.1). CP values were in accordance with those reported in Gonthier et al. (2004). SCP showed a marked decrease ($P < 0.05$) upon autoclaving, but the change appeared to plateau for the two longer treatments which were not significantly different from one another. The decrease in SCP was mirrored by the results for micronized flaxseed

reported by Gonthier et al. (2004), as well as for autoclaved sunflower in Mustafa et al. (2003b). It is of interest to note that upon extrusion, the amount of SCP in flaxseed increased (Gonthier et al. 2004, Mustafa et al. 2003a), which is the opposite of the observed effect of autoclaving. The difference is likely caused by the physical aspect of extrusion which would lend itself to abrogating the oil/protein matrix more readily, thus possibly leading to an increase in both EE and SCP. The oil/protein matrix is physically different than a starch/protein matrix, as part of the oil/protein matrix is most likely in a liquid form, which may also account for the differences observed when extruding oilseeds vs. cereals. NPN did not show any significant increase with the exception of the third treatment where NPN increased ($P < 0.05$) from 1.4 to 2.0 (%DM). These results differ from those of Mustafa et al. (2003b), where for autoclaved sunflower seed they reported a decrease in NPN as treatment time increased, yet upon extrusion NPN in flaxseed has been shown to increase (Mustafa et al. 2003a). NDIP showed a similar pattern to NPN, in that only the 1-h treatment was significantly different ($P < 0.05$) from the raw samples and it increased ($P < 0.05$) from 3.8 to 5.7 (%DM). This is also in accordance with NDIP results for micronized flaxseed reported in Gonthier et al. (2004), as well as with results for autoclaved sunflower seed in Mustafa et al. (2003b). ADIP results showed a small incremental increase between each treatment, increasing from 0.5 (%DM) for raw flaxseed to 0.8 (%DM) for the flaxseed from the 1-h treatment. From these results for the N fractions of flaxseed, it is clear that autoclaving as a form of heat treatment is somehow different from extrusion, likely due to the physical changes that extrusion imparts to the feed sample.

Results for the CNCPS CP sub-fractions are presented in Table 4.3. Fraction PA, representing NPN, was significantly higher only for the 1-h autoclave treatment. Fraction PB1 was the only fraction to decrease ($P < 0.05$) in concentration with autoclave treatment, with each treatment showing a significant difference from the next and when compared to the raw samples, treatments dramatically reduced PB1 from 45.9 (% CP) to 10.5 (%CP). PB1 degrades in the rumen so it would follow that the autoclave treatments would decrease the overall degradation rate of protein in the rumen given that changes in PA were not large enough to offset the changes in degradability caused by the decrease in PB1. Results for the PB1 fraction changes are similar to those reported for autoclaved sunflower seed (Mustafa et al. 2003b). The PB2 fraction was increased ($P < 0.05$) by autoclave treatment, reaching a plateau of 73 (%CP) for the 1-h treatment from a basis of 43 (%CP) for the raw flaxseed. The increase observed in this fraction upon autoclaving would account for the majority of the loss in the PB1 fraction, hence it would be expected that the overall degradation rate would decrease upon autoclaving as a result of the shift from the rapidly ruminally degraded PB1 fraction to the slowly ruminally degraded PB2 fraction. The PB3 fraction was shown to increase after autoclave heating, but only the flaxseed exposed to the 1-h treatment was demonstrably different ($P < 0.05$). The increase in the PB3 fraction from 3.3 for raw flaxseed to 4.9 (%CP) for the 1-h treatment, although statistically significant, is not likely to a major factor in the reduction of ruminal degradability that was be observed upon autoclaving, as this is a relatively small shift. This is despite the fact that it is more slowly degraded in the rumen than fraction PB2. Fraction PC is not considered degradable in the rumen and shows a gradual progression upwards with increasing autoclave treatment time, increasing from 1.9 (%CP) for the raw

Table 4.3 CNCPS protein sub-fraction profile of the raw (control) compared to the autoclave treated Vimy flaxseed.

		Autoclave Treatment (120°C)			
Sub-Fraction	Control	20 min	40 min	60 min	SEM
Crude Protein (%DM)	23.9	23.7	23.8	23.8	0.13
PA (%CP)	6.0^{b}	5.3^{b}	6.4^{b}	8.5^{a}	0.41
PB1 (%CP)	45.9^{a}	30.7^{b}	16.5^{c}	10.5^{d}	0.99
PB2 (%CP)	43.0^{c}	58.3^{b}	71.0^{a}	73.3^{a}	1.09
PB3 (%CP)	3.3^{b}	3.5^{b}	3.7^{b}	4.9^{a}	0.15
PC (%CP)	1.9^{c}	2.2^{bc}	2.5^{ab}	2.8^{a}	0.11
True Protein (%CP)[1]	92.2^{a}	92.6^{a}	91.1^{a}	88.7^{b}	0.46
PB1 (%TP)	49.8^{a}	33.2^{b}	18.1^{c}	11.8^{d}	1.21
PB2 (%TP)	46.6^{d}	63.0^{c}	77.9^{b}	82.6^{a}	1.10
PB3 (%TP)	3.6^{b}	3.8^{b}	4.0^{b}	5.6^{a}	0.16

[a, b, c, d] Means with the same superscripts in the same row are not significantly different ($P < 0.05$) Means separated using the LSD method

SEM = Standard error of mean

(PA = non protein nitrogen, PB1 = soluble true protein fraction, PB2 = insoluble true protein not bound to fibre, PB3 = insoluble true protein bound to fibre, PC = unavailable protein)

[1]True Protein (%CP) = PB1 (%CP) + PB2 (%CP) + PB3 (%CP)

to 2.8 (%CP) for the 1-h autoclave treatment. The protein in this fraction would be bound to other feed constituents such as lignin and tannins, and as such it would not be available to the animal (Sniffen et al. 1992). The miniscule shift observed in this fraction suggests that the autoclave treatments used in this experiment would not be detrimental to the protein quality of flaxseed in terms of its use as a feed ingredient.

As shown in Table 4.4, autoclave heating had no significant effect on the DM degradation rate of flaxseed, nor did it have any effect on the DM lag time as determined in this study. The soluble fraction of DM demonstrated some interesting results, with the 20-min autoclave treatment causing a significant reduction ($P < 0.05$) in the solubility of DM, whereas the 1-h autoclave treatment caused a numerical increase in the soluble fraction of DM. Mustafa et al. (2003b) also demonstrated a decrease in DM degradation upon autoclaving sunflower seed. However, despite the temperatures being similar, the longest treatment in that study was only 30 min, which may not have been long enough to demonstrate the pattern seen in the present study. The decrease in soluble DM was also accompanied by a decrease in soluble CP, which would be responsible for a portion of the change in soluble DM. In a separate study by Mustafa et al. (2003a) using extruded flaxseed, an increase in soluble DM was reported when compared to a raw sample. This increase was also accompanied by an increase in soluble CP. This study showed a similar pattern for soluble CP and DM, suggesting that the majority of the change in the S fraction of DM between the treatments is largely the result of changes to the S fraction of CP. The undegradable fraction of DM increased ($P < 0.05$) upon autoclaving from 12.7 (%DM) to 20.6 (%DM), but there were no significant differences between the treatments. The potentially degradable fraction of DM decreased ($P < 0.05$) from 64.5 (%DM) to

Table 4.4 *In situ* degradation parameters of dry matter for the raw (control) compared to the autoclave treated Vimy flaxseed.

		Autoclave Treatment (120°C)			
	Control	20 min	40 min	60 min	SEM
K_d (%/h)	6.8	7.5	7.8	6.0	0.98
T0 (h)	0.5	0.5	0.0	0.0	0.25
S (%DM)	22.8^{ab}	15.2^{c}	18.6^{bc}	25.6^{a}	2.66
U (%DM)	12.7^{b}	19.1^{a}	20.6^{a}	19.0^{a}	0.90
D (%DM)	64.5^{a}	65.8^{a}	60.9^{ab}	55.4^{b}	2.12
EDDM (%DM)	57.1^{a}	51.4^{b}	52.7^{b}	53.2^{b}	0.73
BDM (%DM)	42.9^{b}	48.6^{a}	47.3^{a}	46.8^{a}	0.73

[a, b, c, d] Means with the same superscripts in the same row are not significantly different ($P < 0.05$). Means separated using the LSD method

SEM = Standard error of mean

(K_d = degradation rate, T0 = lag time, S = soluble fraction, U = undegradable fraction, D = degradable fraction, EDDM = effective degradability of dry matter, BDM = bypass dry matter)

55.4 (%DM). The only treatment to be significantly different from the raw sample was the 1-h treatment. These results are in contrast to those reported by Mustafa et al. (2003b) for autoclaved sunflower seed. The effectively degradable DM also decreased upon autoclave treatment, however, there were no differences between the treatments. Once again, this trend was mirrored in the Mustafa et al. (2003b) study which showed a decrease in the effectively degradable DM of sunflower seed. The opposite effect was observed for flaxseed extrusion in the Mustafa et al. (2003a) study.

The degradation rate of CP upon autoclaving flaxseed decreased ($P < 0.05$) from 7.2 %/h to 5.5 %/h as shown in Table 4.5. The only treatment significantly different from the raw samples in this respect was the 1-h treatment. Decreases in CP degradation rates have also been reported for extruded flaxseed (155°C with a 43 sec retention time) (Mustafa et al. 2003a), autoclaved sunflower seed (127°C, 117 kPa, for 10, 20 and 30 min) (Mustafa et al. 2003b) and roasted sunflower seed (250°C for 60 sec) (Sarrazin et al. 2003), so in this respect the K_d of CP from full fat oilseeds appears to respond consistently to heating despite the method of heating used. No differences were observed for the lag time of CP in the present study. The soluble fraction of CP showed an interesting response where the shortest treatment exhibited the largest decrease, from 24.3 to 12.6 (%CP) and the longer the autoclave treatment, the less it decreased the soluble fraction. Considering other full-fat oilseeds, upon extrusion flaxseed showed an increase in the soluble fraction of CP (Mustafa et al. 2003a), but a decrease was observed for roasted and autoclaved sunflower seed (Mustafa et al. 2003b, Sarrazin et al. 2003). Here, physical changes imparted to the oilseed as a result of processing may be responsible for the difference in response. The undegradable CP fraction in this study increased ($P < 0.05$) with increasing treatment

Table 4.5 *In situ* degradation parameters of crude protein for the raw (control) compared to the autoclave treated Vimy flaxseed.

		Autoclave Treatment (120°C)			
	Control	20 min	40 min	60 min	SEM
K_d (%/h)	7.2[a]	6.1[ab]	6.1[ab]	5.5[b]	0.48
T0 (h)	0.9	0.6	0.0	0.7	0.37
S (%CP)	24.3[a]	12.6[c]	14.2[bc]	18.4[b]	1.72
U (%CP)	6.5[c]	13.3[b]	17.0[a]	16.6[a]	1.07
D (%/h)	69.2[ab]	74.2[a]	68.8[ab]	64.5[b]	2.10
EDCP (%CP)	62.0[a]	49.7[b]	48.9[b]	49.2[b]	1.09
BCP (%CP)	38.0[b]	50.3[a]	51.1[a]	50.8[a]	1.09
EDCP (%DM)	14.4[a]	11.6[b]	11.4[b]	11.4[b]	0.27
BCP (%DM)	8.8[b]	11.8[a]	11.9[a]	11.8[a]	0.25

[a, b, c, d] Means with the same superscripts in the same row are not significantly different ($P < 0.05$). Means separated using the LSD method

SEM = Standard error of mean

(K_d = degradation rate, T0 = lag time, S = soluble fraction, U = undegradable fraction, D = degradable fraction, EDCP = effective degradability of crude protein, BCP = bypass crude protein)

time. With the raw flaxseed having an undegradable CP fraction consisting of 6.5 (%CP), the first 20-min treatment doubled this fraction to 13.3 (%CP) and the 40-min treatment almost tripled this fraction to a value of 17.0 (%CP) where it appeared to reach a plateau. The potentially degradable fraction showed an interesting response where the 20-min autoclave treatment increases it ($P < 0.05$) from 69.2 (%CP) to 74.2 (%CP), and the 40-min and 1-h treatments did not significantly change the potentially degradable CP fraction. However, it should be noted that the raw samples were not significantly different from the treatments and only 20-min and 1-h treatments were significantly different from one another. Presumably there is a point where no change in the degradable fraction as determined by this study would be observed under these conditions. Finally, the effective degradability of CP decreased ($P < 0.05$) from 62.0 (%CP) to approximately 49.2 (%CP), with none of the treatments found to be significantly different from one another. The same response to effective degradability of CP was found for roasted full-fat sunflower seed (Sarrazin et al. 2003) and autoclaved sunflower seed (Mustafa et al. 2003b), while once again extruded flaxseed exhibited the opposite response (Mustafa et al. 2003a).

Results from the three-step *in vitro* estimation of intestinal digestibility are presented in Table 4.6. No difference ($P < 0.05$) was detected between the raw and the autoclave treatments. It is possible that the temperature or the duration of the treatments was not sufficient to damage the flaxseed protein sufficiently for there to be any detectable differences via this method. In the study outlining the protocol (Calsamiglia and Stern, 1995), soybean meal was used and a decrease in digestibility was only observed after heating for 2.5 h at a temperature of 165°C. This is clearly a much more extreme heat treatment than the one used in the present study. What these results suggest is that the

Table 4.6 Comparison of intestinal protein digestibility results for the raw (control) compared to the autoclave treated Vimy flaxseed.

		Autoclave Treatment (120°C)			
	Control	20 min	40 min	60 min	SEM
Intestinal protein digestibility (% CP in ruminally incubated residues)	61.7	58.6	58.4	60.3	1.94

[a, b, c, d] Means with the same superscripts in the same row are not significantly different ($P < 0.05$). Means separated using the LSD method

SEM = Standard error of mean

digestibility of autoclaved flaxseed protein is not adversely affected when heated within the scope of the treatments used in this trial. However, the digestibility results for flaxseed were much lower, 60% vs. 95%, than those previously published for soybean meal (Calsamiglia and Stern, 1995). As such, it should be possible to increase RUP without any accompanying decrease in digestibility in the small intestine.

The results for predicting protein supply to the small intestine are presented in Table 4.7. MCP results showed no difference between the autoclave treatments, where MCP decreased ($P < 0.05$) from 12.38 (% DM) for the raw flaxseed to 9.58 (% DM) for the 1-h treatment. The change in MCP observed is attributable to the decrease in RDP observed upon autoclaving. Results are in accordance with those reported by Yu et al. (2004c) for roasted white lupine. ECP results showed small changes ($P < 0.05$) between the treatments and the raw samples. The changes observed were simply a consequence of changes in the DM% that occurred in flaxseed samples upon autoclaving, increasing from 1.08 (% DM) to 1.10 (% DM). This is because the NRC model estimates ECP from DM content. An increase in DM% upon heating is routinely observed when heating feeds by various means (Chang et al. 1987, Yu et al. 2000) so this is not very surprising. RUP increased ($P < 0.05$) from 9.36 (% DM) for raw flaxseed to 12.54 (% DM) for the 1-h autoclave treatment with no statistically discernable difference observed between the treatments. When the values for MCP and RUP are taken into account, they offset one another, leaving the value for flaxseed MP unaffected by autoclave treatment in this study. The degraded protein balance (DPB) estimate for all flaxseed samples was negative, which suggests a potential shortage of N in the rumen in terms of the available energy provided by the flaxseed. There was no significant difference in DPB for any of

Table 4.7. Results from the NRC (2001) Nutrient Requirements for Dairy Cattle model predicting protein supply to the small intestine for raw (control) compared to autoclave treated Vimy flaxseed.

		Autoclave Treatment (120°C)			
(% DM)	Control	20 min	40 min	60 min	SEM
Microbial CP	12.38[a]	9.79[b]	9.59[b]	9.58[b]	0.200
Absorbable Microbial CP	7.93[a]	6.27[b]	6.14[b]	6.13[b]	0.128
Endogenous CP	1.077[c]	1.099[b]	1.102[a]	1.102[a]	0.0005
Absorbable Endogenous CP	0.430[c]	0.440[b]	0.441[a]	0.441[a]	0.0002
Ruminally undegraded CP	9.36[b]	12.14[a]	12.50[a]	12.54[a]	0.194
Absorbable Ruminally undegraded CP	5.77[b]	7.13[a]	7.31[a]	7.56[a]	0.268
Metabolizable CP	14.12	13.83	13.89	14.14	0.202
[1]Degraded Protein Balance	-3.99[a]	-7.54[b]	-7.83[b]	-7.80[b]	0.241

[a, b, c, d] Means with the same superscripts in the same row are not significantly different ($P < 0.05$). Means separated using the LSD method

SEM = Standard error of mean

[1]Degraded protein balance for NRC-2001 calculated according to Yu et al. 2003.

the autoclave treatments. However, the DPB shifted ($P < 0.05$) from -3.99 (% DM) for raw flaxseed to -7.83 (% DM). A portion of the difference between the raw and the autoclaved flaxseed sample DPBs arises from RDP and how TDN is calculated according to the NRC (2001) formula, as well as from the fact that the EE fraction of the flaxseed samples increased ($P < 0.05$) upon autoclave treatment.

The results from modeling nutrient supply to the small intestine using the DVE/OEB model are presented in Table 4.8. The DVE/OEB system takes into consideration some factors not considered by the NRC (2001) model, which generate some slightly different values for roughly the same feed parameters. FOM results showed decreases ($P < 0.05$) for the autoclaved treatments. FOM consists of digestible organic matter corrected for the fat contained in the feed, which is not considered degradable in the rumen, as well as RUP, so in light of the EE fraction and RUP fraction increasing upon autoclaving, these results are to be expected. FOM has been shown to decrease for several different feedstuffs upon heating (Yu et al. 2002). In the DVE/OEB system, changes in FOM are responsible for the calculated changes in MCP and AMCP. Endogenous CP was reduced ($P < 0.05$) for flaxseed upon autoclaving from 1.70 (% DM) to 1.47 (% DM). Ruminally undegraded feed protein, as in the NRC (2001) model, showed a significant increase ($P < 0.05$) upon autoclaving from 10.39 (% DM) to 13.92 (% DM), with no significant differences between the autoclave treatments. Absorbable protein in the intestine, DVE under the DVE/OEB model, was increased ($P < 0.05$) upon autoclaving flaxseed. In this case, the relative increase of RUP was greater than the decreases observed for AMCP, AECP and ARUP, so the results from the treatments was for RUP to increase ($P < 0.05$) from 6.96 (% DM) for raw flaxseed to 8.97 (% DM) for the flaxseed exposed to the

Table 4.8. Results from the DVE/OEB system predicting protein supply to the small intestine for the raw (control) compared to the autoclave treated Vimy flaxseed.

		Autoclave Treatment (120°C)			
(% DM)	Control	20 min	40 min	60 min	SEM
Fermentable Organic Matter	23.61[a]	19.83[c]	19.67[c]	21.40[b]	0.337
Microbial CP	3.54[a]	2.97[c]	2.95[c]	3.21[b]	0.051
Absorbable Microbial CP	2.26[a]	1.90[c]	1.88[c]	2.05[b]	0.032
Endogenous CP losses	1.70[a]	1.62[b]	1.57[c]	1.47[d]	0.0002
Ruminally undegraded CP	10.39[b]	13.47[a]	13.87[a]	13.92[a]	0.215
Absorbable Ruminally Undegraded CP	6.40[b]	7.91[a]	8.11[a]	8.39[a]	0.297
Absorbable Protein (DVE)	6.96[c]	8.19[b]	8.42[ab]	8.97[a]	0.288
Degraded Protein Balance (OEB)	10.00[a]	7.21[b]	6.96[b]	6.68[b]	0.216

[a, b, c, d] Means with the same superscripts in the same row are not significantly different ($P < 0.05$). Means separated using the LSD method. SEM = Standard error of mean

autoclave treatment for 1 h. The OEB values for all treatments and the raw flaxseed were determined to be positive without any significant differences between the treatments. The OEB value was decreased by autoclave treatment, suggesting that the potential loss of N from the rumen was mitigated when the flaxseed was autoclaved. However, according to the model there would still be significant potential N losses from the rumen due to a lack of available energy for BPS (Tamminga et al. 1994). Conversely, there would be excessive amounts of nitrogen in the rumen relative to the energy available for the bacteria to convert it into protein and some of that nitrogen could be lost to the animal.

In comparing some of the key values of the NRC (2001) model to the DVE/OEB model, there are some notable differences. The most striking is the difference in the DPB values provided by each model. In the case of the NRC (2001) model, DPB values are all negative. This signifies that a potential N deficiency exists in the rumen and that MCP production in the rumen would be impaired as a consequence. In the DVE/OEB system, a positive balance was observed, suggesting excess N in the rumen and therefore potential N loss. Also of interest is the degree to which these values differ where the NRC (2001) model and the DVE/OEB system are reasonably close in magnitude but have opposite signs. This difference arises primarily from the fact that the NRC (2001) based method used in the present study was based on a TDN approach to calculate MCP, which considers the EE fraction in its calculation, whereas the FOM approach in the DVE/OEB system eliminates the EE fraction in the feed from the calculation as it is not considered ruminally degradable. The TDN results are affected further by the EE fraction, because in the conversion of digestible fatty acids to TDN, the digestible fatty acid value is multiplied by 2.5. Because flaxseed contained roughly 42% EE on a DM basis for raw

flaxseed and 44% EE on a DM basis for the autoclaved treatments, this would have serious implications for the model when assessing MCP based on the TDN method. Because of the differences in MCP estimation, the differences in the results that were observed are logical, because the NRC (2001) TDN based method considers that there is much more energy in the equation. It would follow then that more N would be required in the rumen for MCP production to be in balance. In this instance, it appears as though the DVE/OEB system provides us with a more reliable estimate of how much metabolizable protein is available from high fat feeds.

In this study, the TDN based MCP results may not be accurate. This is due in large part to variation in the efficiency of MCP synthesis. The variation must be accounted for to provide sufficient accuracy when using indicators of fermentable energy such as, TDN (NRC 2001). In the case of flaxseed, differences in MCP efficiencies would be expected due to the elevated oil content. Modeling a total mixed ration with flaxseed would provide a better indication of its consequences on the diet, but would not permit the direct comparison of the two models used in the present study. The effects on MCP efficiency are not as significant in the DVE/OEB system because only those feed elements that are fermented in the rumen are part of FOM (Tamminga et al. 1994), but in this case no accounting of the effects the fat would have on MCP production is done.

4.4 Conclusions

Autoclave treatments used in the present study were effective at manipulating the nutrient composition of flaxseed. Autoclaving induced changes in flaxseed that in most respects matched those of roasting or micronization, as opposed to extrusion which appeared in

many instances to cause effects opposite to those achieved by other heat treatments. It is possible that this discrepancy is the result of the actual physical aspects of extrusion-based heat treatments. Generally speaking, nutrient degradability was decreased upon autoclave treatment, and the site of nutrient digestion for DM and CP was shifted to the small intestine. The autoclave treatments employed in this study were not totally distinguishable in terms of many of the feed parameters measured, and it is likely that much more extreme treatments in terms of temperature and time would be required for this not to be the case. From the three-step *in vitro* intestinal protein digestibility results, it can be further concluded that the shifts in digestion location are not accompanied by a concomitant decrease in protein degradability, which would make the autoclave treatments used in this study acceptable for this specific purpose although not necessarily optimal.

From the protein supply modeling results, it is clear that caution must be used while employing these models the evaluation of oilseeds. In this study, modeling was done with a single feed, flaxseed, so using a total ratio might provide different results. Oilseeds are not meant to constitute large portions of the ruminant diet and these models were developed with other feedstuffs comprising the majority of the diet. The comparison between the NRC (2001)-derived DPB and the DVE/OEB system illustrates this fact quite noticeably. The difference is striking when compared to the differences in the results from the models found for other feeds, the disparity between the two models is logical when their formulae are taken into account with feed composition.

The results obtained that pertain to protein degradability suggest that sufficient chemical and structural changes were induced in flaxseed protein upon autoclaving to alter its

chemical properties. These results permit probing the flaxseed with DRIFT and S-FTIR techniques to reveal inherent structural changes in the molecular spectra that are associated with the autoclaving process and its effects on the process of digestion.

5. DETECTING MOLECULAR CHANGES IN FLAXSEED PROTEIN SECONDARY STRUCTURE USING DRIFT AND S-FTIR SPECTROSCOPIC TECHNIQUES

5.1 Introduction

Current methods of feed evaluation, particularly for protein quality, are typically slow and cumbersome processes that require the destruction of a sample for any given measurement. The information gleaned from such procedures is also not completely in context, as some pertinent information is lost by the process of taking the measurements. Part of the reason for this is that these standard methods destroy any spatial information as well as the distribution of those elements of interest (Budevska 2002). These elements may be linked to degradability and digestibility in animals.

Although not novel, vibrational spectroscopy or mid-infrared spectroscopy has the capacity to overcome some of the issues with modern feed characterization methods. The results from mid-IR spectroscopy are complex in nature. The information contained within each spectrum is related not only to the chemical bonds in each sample but interrelationships between these chemical bonds (Novikov et al. 1998). So, rather than look at the components of interest separate from one another, it is possible to look at everything in context. When a synchrotron is used as the source of IR light, it is known as synchrotron based Fourier transform infrared microspectroscopy (S-FTIR). This coupling permits an even greater amount of information to be gathered from a sample of interest at both the cellular and molecular levels. The qualities of synchrotron light, including brightness and source size, permit IR spectroscopic measurements to be made on a sub-

cellular scale, thus allowing us to link relative quantities, interrelationships and localization of compounds of interest all in a single experiment (Wetzel and Levine, 2001). This mid-IR technique permits a greater understanding of feed structures at the sub-cellular level (Yu 2004), and with further development will allow a more complete characterization of those feed traits related to quality.

One feature of protein that can be quantified with mid-IR methods is its secondary structure, which may have some links to protein digestibility (Yu et al. 2004a). This is because digestion is a complex process where any kind of steric issues at the molecular level, such as those presented by the different secondary structures, will ultimately affect the access enzymes will have to the feed constituents. Heating has also been demonstrated to alter the relative amounts of each secondary structure, thus having possible connections to alterations in degradability (Yu 2005c).

The objective of this study was to use both diffuse reflectance infrared Fourier transform spectroscopy (DRIFT) and S-FTIR as a means of detecting heat induced changes to feed and feed proteins. Using multivariate molecular spectral analysis techniques, as well as measuring α-helix to β-sheet sheet ratios, should allow us to identify molecular differences between different autoclaved flaxseed treatments. The goal was to attempt to understand and identify spectroscopic signatures that are associated with differences in ruminal degradability. The hypothesis of this study was that autoclaving would induce changes in flaxseed mid-IR spectra that can be characterized with DRIFT and S-FTIR.

5.2 Materials and Methods

5.2.1 Flaxseed heating and processing

Flaxseed (*Linum usitatissimum* L. cv. Vimy) grown at Moose Jaw, Saskatchewan during the 2005 growing year was provided by Shamrock Seeds Ltd. Saskatoon, Saskatchewan. Three-kilogram samples of flaxseed were heated by autoclave (Amsco Eagle SG-3031, STERIS Corporation, Mentor, Ohio USA) at 120°C in gravity mode for 20, 40 or 60 min. Treatments were then repeated on a separate set of 3-kg samples providing two sets of treatments, A and B. Control treatments were unheated. Samples were subsequently cooled to room temperature and then placed in the refrigerator prior to grinding.

5.2.2 Diffuse Reflectance Fourier Transformed Infrared Spectroscopy (DRIFT)

Sub-samples of the cooled flaxseed batches were ground with a coffee grinder (Braun KSM 2, Proctor and Gamble Inc., Toronto, ON, CAN), chilled again and re-ground. Grinding was done for 3 min each time. The cooling was done so as to prevent the samples from forming dough and from getting too warm, thus creating artifacts in the spectra. The coffee grinder was selected to minimize the effects of grinding on the flaxseed spectra, ensuring the physical cracking and breaking of the sample yet maintaining as much of the smaller plant seed structures as possible. Samples of the ground flaxseed were then mixed with KBr in a ratio of 4 parts flaxseed to 1 part KBr in a 2 mL centrifuge tube and mixed by vortex for several min. Raw flaxseed was used for control samples. DRIFT was performed using a Bio-Rad FTS-40 with a ceramic IR source and MCT detector (Bio-Rad laboratories, Hercules, CA, USA). Data was collected using Win-IR software. Spectra were generated from the mid-IR (4000-800 cm^{-1}) portion

of the electromagnetic spectrum with 256 co-added scans and a spectral resolution of 4 cm^{-1}. Spectral analysis was done with OMNIC 6.0 (Thermo Nicolet, Madison, WI, USA). Eight spectra were collected per treatment.

5.2.3 S-FTIR window preparation

Randomly selected whole flaxseeds from treatment samples were sectioned to a thickness of 6 µm using a microtome (Microm 330 (Microm Laborgerate GmbH, Sandhausen, GER) at the Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, SK, Canada. The detailed preparation procedure was reported in Walker (2007). Sample sections were subsequently transferred to Barium Fluoride (BaF_2) discs (Spectral Systems, Hopewell Junction, NY, USA; part number 915-3015). Each BaF_2 disc had three separate sections from three separate flaxseeds placed on it. Fourteen seeds per treatment were analyzed. At least ten spectra from randomly selected spots within the cotyledons of each seed were generated. More spectra were gathered when a collected spectrum was considered to be excessively noisy for analysis.

5.2.4 S-FTIR microspectroscopy

S-FTIR microspectroscopy was performed on beam line U2B at the National Synchrotron Light Source located at the Brookhaven National Laboratory (NSLS, BNL, Department of Energy, Upton, NY, USA). Collimated light from the synchrotron was directed to an FTIR spectrometer (Nicolet Magna 860, Madison, WI, USA) with a KBr beam splitter and a nitrogen cooled mercury cadmium telluride (MCT-A) detector through a port on the spectrometer. The spectrometer was interfaced with a Nic PLAN IR microscope (Nicolet Instruments, Madison, WI, USA) using a single image plane mask set to give an aperture

of 10 µm x 10 µm. Spectra were generated in transmission mode with the mid-IR (4000-800 cm^{-1}) region of the electromagnetic spectrum by 128 co-added scans with a spectral resolution of 4 cm^{-1}. Spectral analysis was done with OMNIC 6.0 (Thermo-Nicolet, Madison, WI, USA) software. Spectra were generated from randomly selected regions within the cotyledons of the flaxseed sections.

5.2.5 Alpha-helix to beta-sheet ratio measurement

Protein secondary structures were estimated using the amide I band located in the region of ca. 1700 cm^{-1} – 1610 cm^{-1} with its peak at ca. 1650 cm^{-1}. Two different methods were used to estimate protein secondary structure concentrations for DRIFT spectra. In the first method, the spectra were first converted by Fourier self-deconvolution with OMNIC 6.0 to identify the component peaks and their frequencies which comprise the amide I band in the spectra. The areas of the component bands representing the amount of α-helix and β-sheet in the FSD spectra were then calculated using Origin 7 (Origin Lab Corporation, Northampton, MA, USA) with both the Gaussian and Lorentzian multi component peak modeling methods. The area under the peak at ca. 1658 cm^{-1} was taken to represent the molecular bonds generating the amide I band in the α-helical conformation. The area under the peak at ca. 1630 cm^{-1} was taken to represent molecular bonds generating the amide I band in the β-sheet conformation. The second method was used for both DRIFT and S-FTIR spectra and involved using the FSD spectra generated with OMNIC 6.0 to identify the region of the absorption spectra representing the respective secondary structures. OMNIC 6.0 was then used to calculate the areas in the absorption spectra representing the α-helices and β-sheets.

5.2.6 Multivariate analysis of protein spectra

CLA and PCA multivariate statistical methods were used to compare the DRIFT spectra of the different treatments to the control samples to determine if there were some underlying structural differences in the spectral information that would permit the identification of spectra belonging to different treatment groups. First, the absorbance spectra were baseline corrected using the automatic baseline correction function of OMNIC 6.0. The spectra were then analyzed together using Statistica 6.0 software (Statsoft, Tulsa, OK, USA). For CLA and PCA, the spectral regions of 2000 cm^{-1} - 800 cm^{-1}, containing most of the mid-IR spectrum, and 1715 - 1485 cm^{-1}, containing the amide I and II regions, were used. CLA results were presented as dendograms, while PCA results were plotted based on the two highest factor scores and plotted as a function of those scores. In each comparison, the eigenvector for factor 1 was plotted against that for factor 2 which accounted for over 99% of the variability in the data.

5.2.7 Statistical analysis

Statistical analyses of protein secondary structure ratios were performed using the MIXED procedure of SAS (version 9.1) (SAS Institute Inc., Cary, NC, USA). DRIFT spectra were analyzed in a completely randomized design using the model Y = mean + treatment + error. S-FTIR spectra were analyzed in a nested experimental design with the model Y = mean + treatment + seed (nested within treatment) + error. Significance was taken as $P < 0.05$.

5.3 Results and Discussion

Results from protein secondary structure analysis based on the DRIFT spectra are presented in Table 5.1. It is important to note that these ratios are based on relative amounts of the secondary structures present. Using an α-helix to β-sheet ratio to assess changes to protein structure involves many considerations. From IR spectroscopy it is not possible to obtain exact values for each secondary structure, but if all sample spectra are treated in the same manner, it is possible to compare the results from one spectrum to another (Yu 2006a). In addition to this, because of different methodologies used between researchers, results from a given feed can vary one study to another unless the samples are prepared identically. This arises from the sensitivity of mid-IR spectroscopy. Selecting different regions of the spectrum and different sizes for those regions around the amide I and II bands, along with different baselines, are all factors that would influence the resulting α-helix to β-sheet ratio (Yu 2006a). There exist technical issues that affect protein secondary structure analysis as well. The amide I band carbonyl group has different sensitivities to absorption depending on secondary structure, the number of bands it contains varies and is usually not known for certain, and, finally, the bands can be varied in shape (Yu 2006a). Even for relative determinations of the α-helix to β-sheet ratio one must be aware of these facts. Despite these issues, the α-helix to β-sheet ratio can be a useful way of exploring differences in protein structure that exist between two samples.

What is clear from Table 5.1 is that all three different ratio measurements show that the ratio of α-helix to β-sheet in flaxseed decreases significantly upon heating. This change is in accordance with results of Yu et al. (2005) using golden flaxseed with roasting as the

Table 5.1. α-helix to β-sheet ratios from DRIFT spectra of the autoclaved whole ground Vimy flaxseed.

	Control	Autoclave Treatment (120°C)			SEM
		20 min	40 min	60 min	
Area under peak Method	2.18[a]	1.60[b]	1.27[c]	1.37[c]	0.011
Multi-comp. peak Modeling (Gauss)	2.08[a]	1.06[b]	0.99[b]	1.23[b]	0.072
Multi-comp. peak Modeling (Lorentz)	2.27[a]	1.07[b]	1.10[b]	1.72[ab]	0.071

[a, b, c, d] Means with the same superscripts in the same row are not significantly different ($P < 0.05$). Means separated using the LSD method

SEM = Standard error of mean

heating method. Of the three methods used to estimate the relative α-helix to β-sheet ratio, only the area under peak method was able to discern any difference between the treatments. Also of interest were the results from the Lorentzian peak fitting method which showed a numerical increase for the 1-h treatment compared to the 20-min and 40-min treatments. These changes in protein secondary structure ratios are likely due to more α-helix structure being denatured or destroyed during the autoclaving process than β-sheet. The Lorentzian data, however, complicates the interpretation and could arise from a plateau in α-helix degradation. The secondary structure analysis also disagrees with results published by Madhusudhan and Singh (1985a, 1985b) who reported that the major proteins of flaxseed, albumin and globulin, consist predominantly of β-sheet secondary structures as determined by the circular dichroism technique using purified protein samples. It is possible, however, that the remaining 14% of protein in flaxseed has extremely high concentrations of α-helix relative to other secondary structures. It should also be noted that there is some degree of variability in terms of oil and protein content within a variety. Growing conditions are known to affect oil and protein content as well. A reduction in the amount of storage protein present in flaxseed would mean that they would contribute less to the overall protein secondary structure profile. The differences between result from this study and other reported values could be explained by the relative concentrations of different proteins.

In Table 5.2, the results of the same analysis are shown for S-FTIR data. The α-helix to β-sheet ratio increased upon heating for 40-min and 1-h in contrast to the DRIFT results. The control sample and the 20-min autoclave treatment were not significantly different from one another as was the case for the 40-min and 1-h treatment samples. One

Table 5.2 α-helix to β-sheet ratio as determined using the area under peak method and S-FTIR data collected from flaxseed cotyledon regions.

		Autoclave Treatment (120°C)			SEM
	Control	20 min	40 min	60 min	
Area under peak Method	0.86b	0.88b	1.03a	1.03a	0.033

[a, b, c, d] Means with the same superscripts in the same row are not significantly different ($P < 0.05$). Means separated using the LSD method

SEM = Standard error of mean

possibility for the difference in the effect autoclaving has on the α-helix to β-sheet ratio between the two mid-IR spectroscopic methods is that DRIFT spectra were from whole flaxseeds. Whole flaxseeds include the seed coat, pericarp, aleurone cells, cotyledons and endosperm. The aleurone cells are rich in protein and their composition may be different from that of the cotyledons. The S-FTIR spectra on the other hand were generated solely from the cotyledons of the flaxseed. Furthermore, it is possible that the individual regions may have different sensitivities to heating, which may cause different changes to secondary structure ratios in different flaxseed tissues. The different regions of the plant would have varying complements of proteins with potentially different responses to heating Further study is required to understand the effect of heating on different tissues within flaxseed and, determining which secondary structure is more resistant to denaturation from heat as well as to degradation- in the rumen.

Figure 5.1 shows the results of cluster analysis for the ca. 2000-800 cm^{-1} spectral region based on DRIFT spectra. Other than a single spectrum from the 20-min autoclaved treatment, the raw flaxseed spectra are grouped separately. The remaining clusters all contain combinations of spectra from the other treatments. This implies that DRIFT spectroscopy combined with cluster analysis can distinguish an autoclaved flaxseed sample from a raw sample and the benefit of cluster analysis itself is that no other prior knowledge of the sample other than a mid-IR spectrum is required to make such a distinction. The cluster analysis comparisons between treatments show similar results to those displayed in Figure 5.1. Figure 5.2 *(top)* shows a comparison between the control spectra and the 20-min treatment spectra. Here a single spectrum from the 20-min treatment is clustered among the control samples. In Figure 5.2 *(bottom*), the 40-min

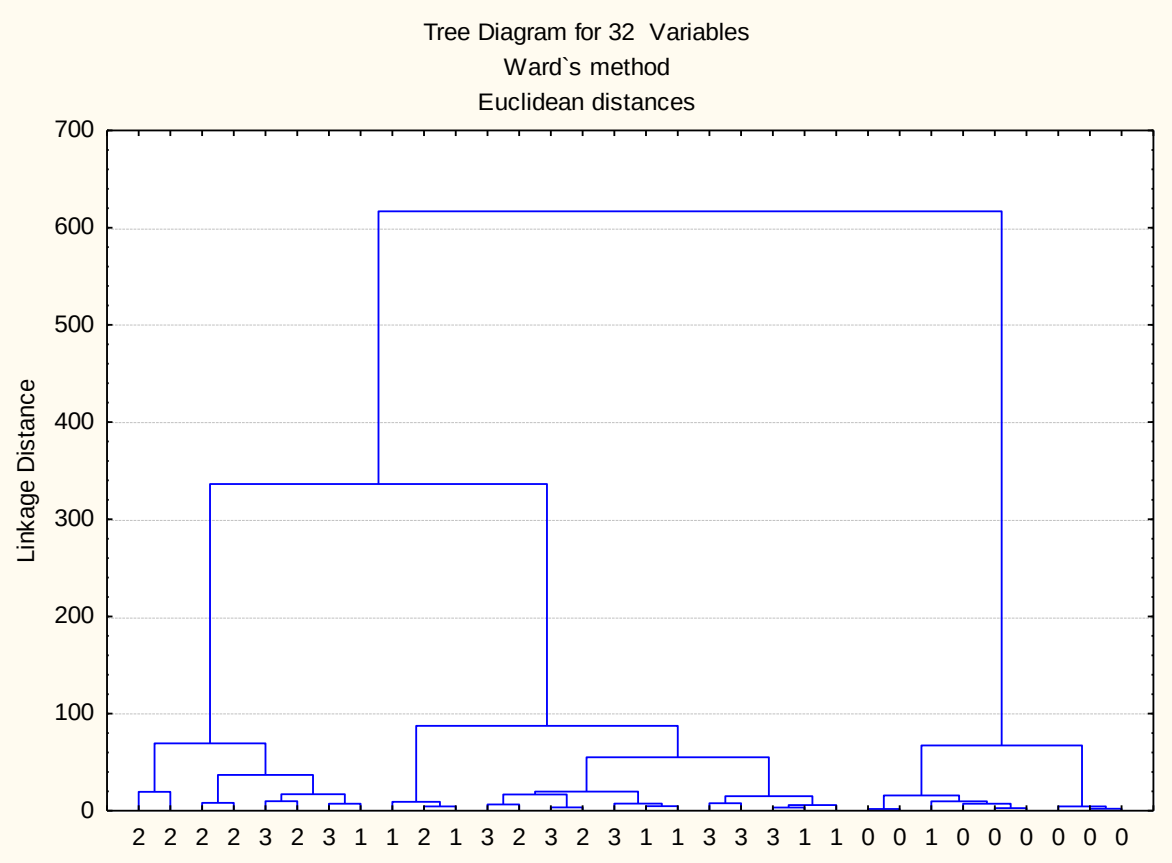

Figure 5.1. CLA analysis of DRIFT based spectra (2000-800 cm^{-1}) obtained from the raw and the autoclaved flaxseed samples (0 = control/raw, 1 = autoclaved @ 120°C for 20 min, 2 = autoclaved @ 120°C for 40 min, 3 = autoclaved @ 120°C for 60 min) [CLA = Hierarchical cluster analysis, DRIFT = Diffuse Reflectance Infrared Fourier Transform]

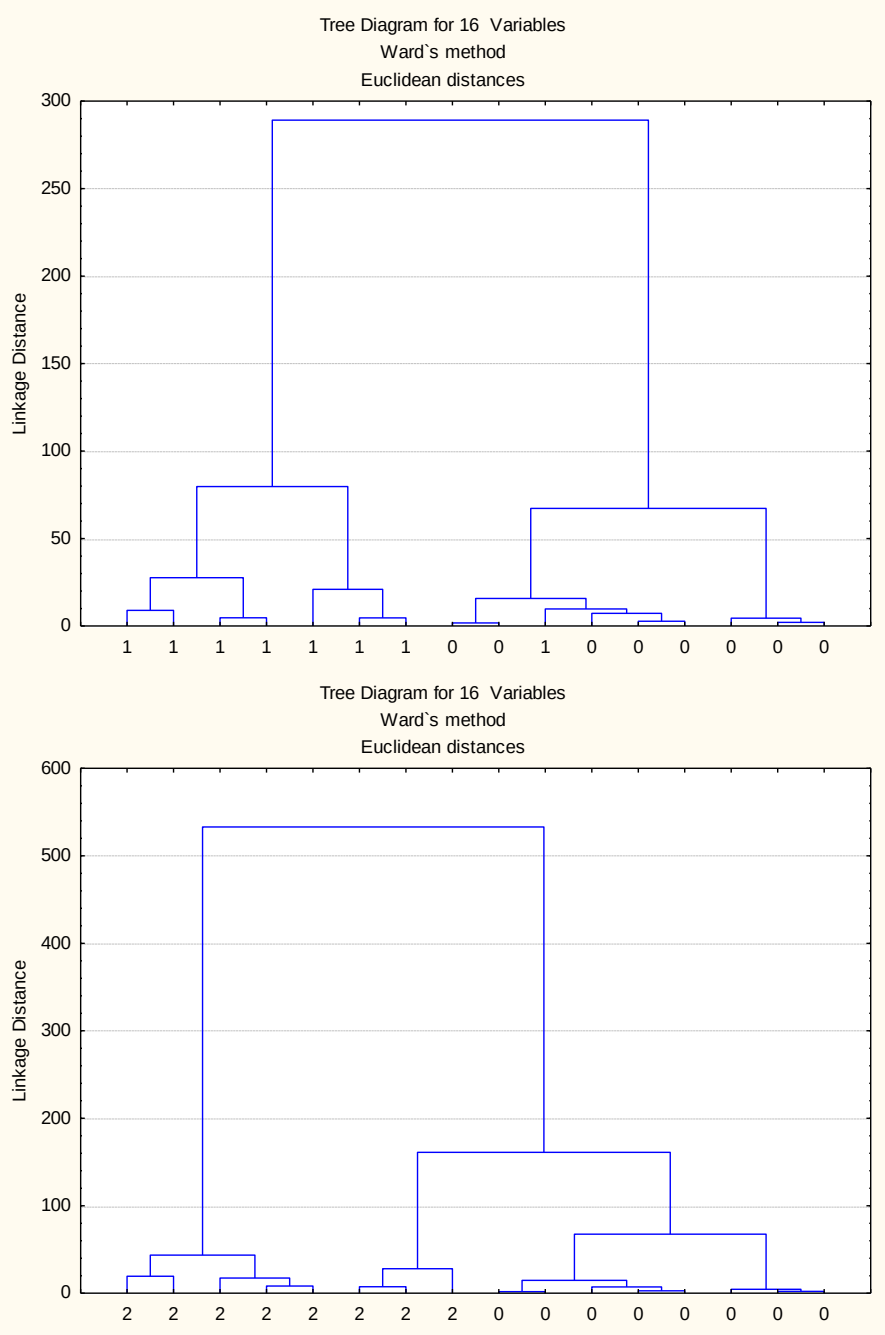

Figure 5.2. CLA analysis of DRIFT based spectra (2000-800 cm^{-1}) obtained from the raw and the autoclaved flaxseed samples. Comparisons of the separate treatments.(*top)* 0 vs. 1, *(bottom)* 0 vs. 2. (0 = control/raw, 1 = autoclaved @ 120°C for 20 min, 2 = autoclaved @ 120°C for 40 min, 3 = autoclaved @ 120°C for 60 min) [CLA = Hierarchical cluster analysis, DRIFT = Diffuse Reflectance Infrared Fourier Transform]

treatment spectra and the control spectra are all found within distinct clusters, although the dendogram shows that some of the 40-min treatment spectra are more closely related to the control spectra. Figure 5.3 *(top)* shows a very clear separation between the control spectra and the 1-h treatment spectra, with all spectra falling within their respective clusters. Figure 5.3 *(bottom*) shows a distinct cluster for the 40-min treatment spectra; however, not all the spectra from the 40-min treatment were similar enough to be assigned to this group. The remaining CLA comparisons (Figure 5.4) failed to demonstrate any distinct groupings between the treatments.

Figure 5.5 shows the results of the PCA conducted using DRIFT spectra. The results show a similar grouping of the raw flaxseed spectra with a single spectrum from the 20-min autoclave treatment. The agreement of both PCA and CLA suggests that there is some similar structure in the data, in this case the baseline corrected spectra, which distinguishes almost completely the raw sample spectra from the spectra of autoclaved samples. The plot was derived from values for the first two principal components generated by the analysis which explain 95.88% and 3.42% of the variability in the spectra. Factor 1 in this case can almost discriminate entirely between the raw and treated flaxseed samples and represents the vast majority of the variability present. As in the case for cluster analysis, Yu et al. (2007) showed that PCA could also distinguish between plant structures, meaning that with little modification of the spectra it is possible to identify which part of a plant a spectrum represents, and also whether or not it has been treated.

Figure 5.6 *(top)* shows the results from PCA between the control and the 20-min treatment spectra. Similar to the CLA results, Figure 5.6 *(top)* shows that a single

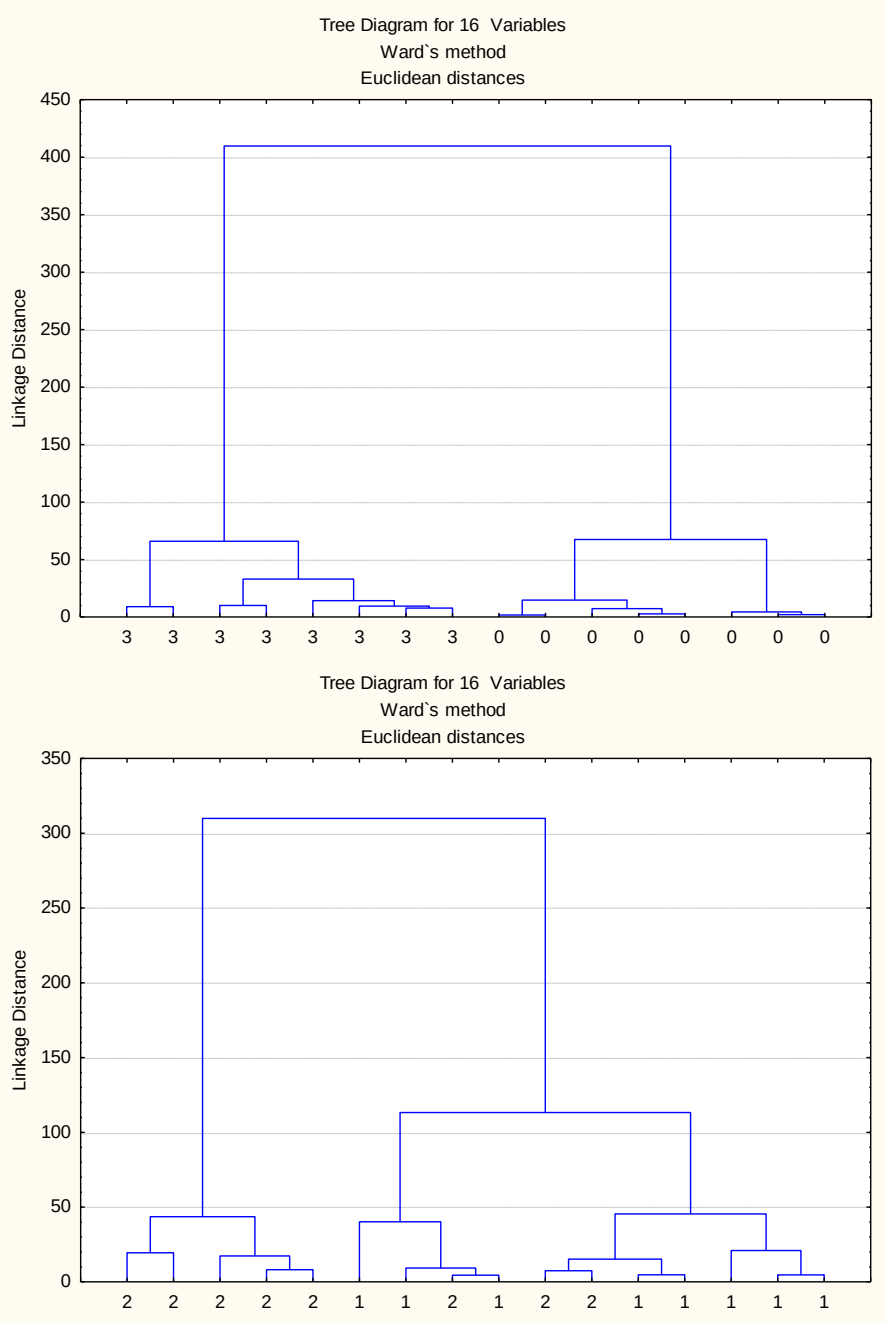

Figure 5.3. CLA analysis of DRIFT based spectra (2000-800 cm^{-1}) obtained from the raw and the autoclaved flaxseed samples. Comparisons of the separate treatments.(*top)* 0 vs. 3, *(bottom)* 1 vs. 2. (0 = control/raw, 1 = autoclaved @ 120°C for 20 min, 2 = autoclaved @ 120°C for 40 min, 3 = autoclaved @ 120°C for 60 min) [CLA = Hierarchical cluster analysis, DRIFT = Diffuse Reflectance Infrared Fourier Transform]

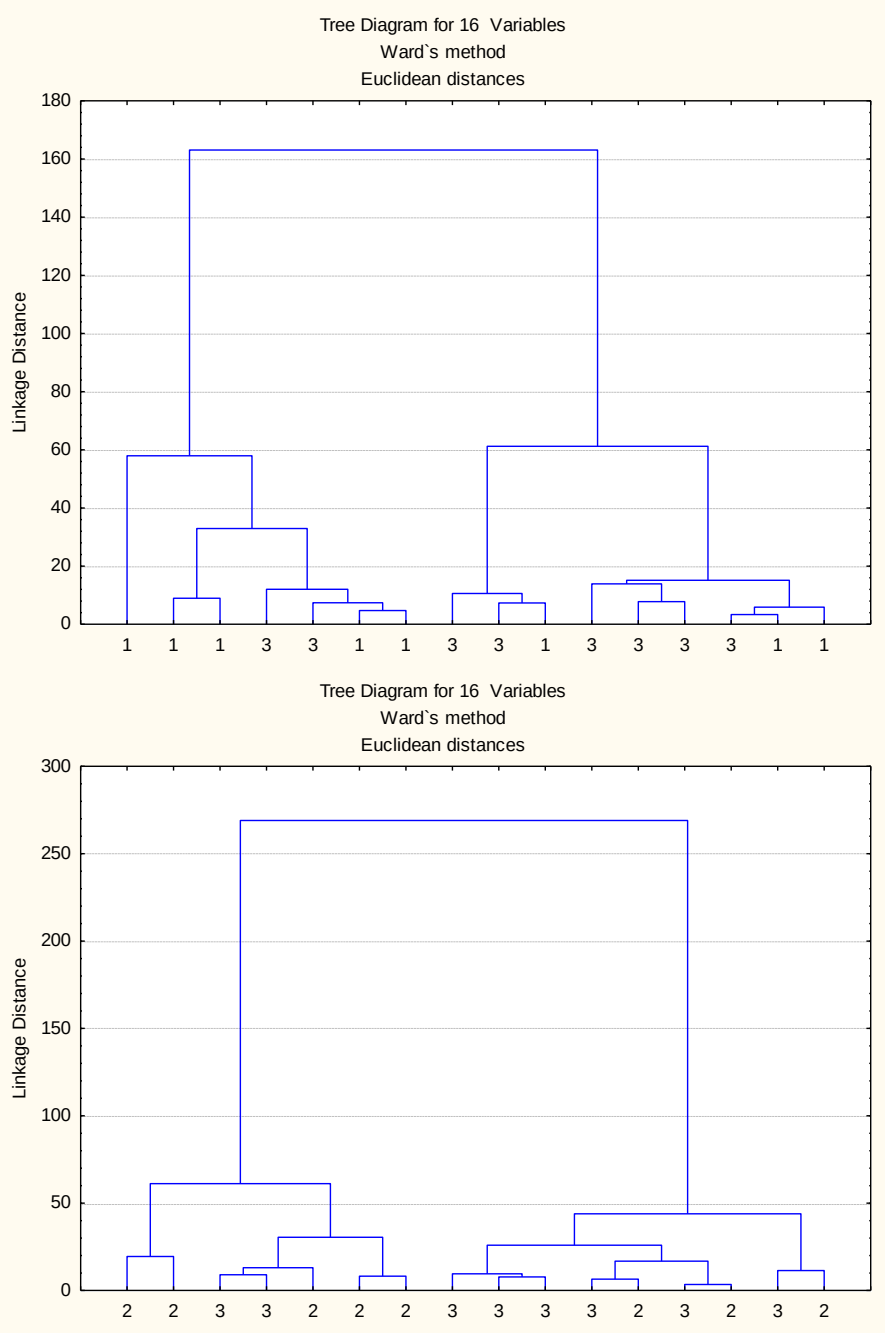

Figure 5.4. CLA analysis of DRIFT based spectra (2000-800 cm^{-1}) obtained from the raw and the autoclaved flaxseed samples. Comparisons of the separate treatments.(*top)* 1 vs. 3, *(bottom)* 2 vs. 3. (0 = control/raw, 1 = autoclaved @ 120°C for 20 min, 2 = autoclaved @ 120°C for 40 min, 3 = autoclaved @ 120°C for 60 min) [CLA = Hierarchical cluster analysis, DRIFT = Diffuse Reflectance Infrared Fourier Transform]

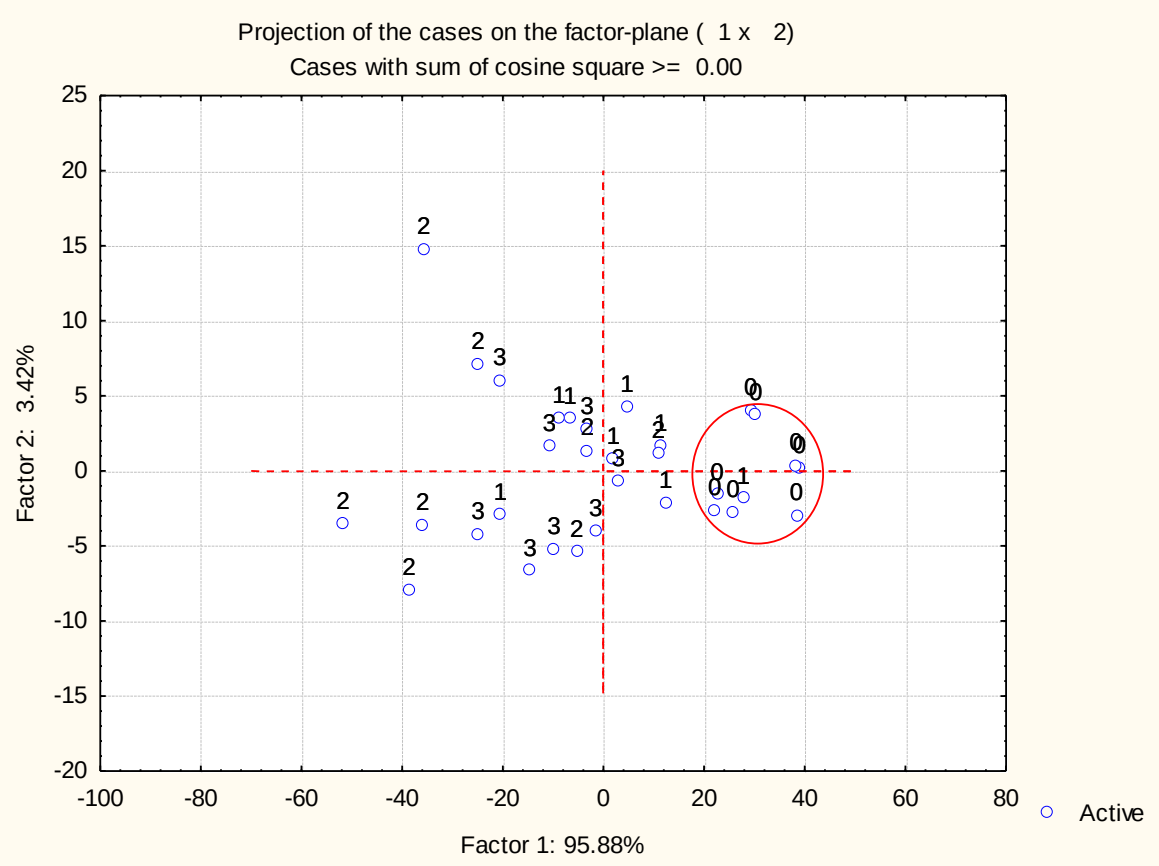

Figure 5.5. PCA analysis of DRIFT based spectra (2000-800 cm^{-1}) obtained from the raw and the autoclaved Vimy flaxseed samples (0 = control/raw, 1 = autoclaved @ 120°C for 20 min, 2 = autoclaved @ 120°C for 40 min, 3 = autoclaved @ 120°C for 60 min) [PCA = Principal components analysis, DRIFT = Diffuse Reflectance Infrared Fourier Transform]

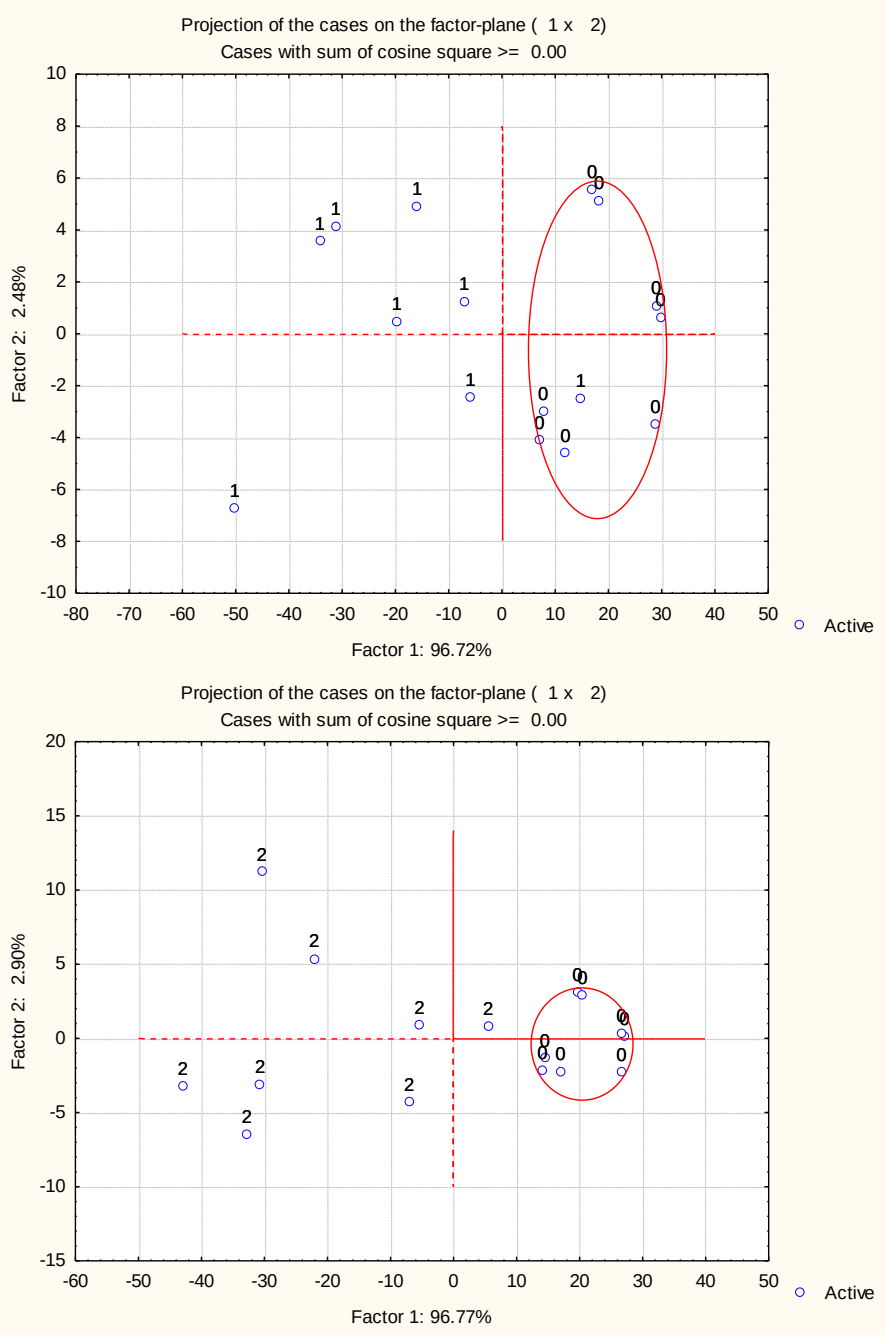

Figure 5.6. PCA analysis of DRIFT based spectra (2000-800 cm^{-1}) obtained from the raw and the autoclaved flaxseed samples. Comparisons of the separate treatments. .(*top)* 0 vs. 1, *(bottom)* 0 vs. 2 (0 = control/raw, 1 = autoclaved @ 120°C for 20 min, 2 = autoclaved @ 120°C for 40 min, 3 = autoclaved @ 120°C for 60 min) [PCA = Principal components analysis, DRIFT = Diffuse Reflectance Infrared Fourier Transform]

spectrum from the first treatment is indistinguishable from the control samples. In Figures 5.6 *(bottom*) and 5.7 *(top),* the control spectra are separated from treatment spectra, although the treatment spectra are spread out. This shows distinction from the CLA results for the 40-min treatment spectra where there was still some linkage to the control samples. The remaining spectral comparisons (Figure 5.7 *(bottom*), Figure 5.8) by PCA were not able to show any clear grouping of the treatment spectra on the factor plane.

CLA and PCA were subsequently conducted on a more defined region of the spectrum, in this case the spectrum from ca. 1715-1485 cm-1 which covered both the prominent amide I and II bands. Figure 5.9 shows the CLA results from this region of the spectrum for all treatment spectra. It shows a similar cluster pattern to that in Figure 5.1, with the exception that within it exists a distinct cluster for the 40-min treatment spectra although not all of the 40-min treatment spectra are contained within it. It also shows that the control samples are found in their own group with a single spectrum from treatment 1. Figure 5.10 *(bottom*) shows CLA results for the comparison of the 40-min treatment to the control spectra. This analysis shows an improvement in the clustering ability when the spectral window was narrowed, in this case both the control spectra and the 40-min treatment spectra are found in their own unique clusters as opposed to when a much larger portion of the spectrum was used. This indicates that the protein structures of the raw flaxseed and those from the 40-min treatment were different. This is similar to the results between raw flaxseed and that from the 1-h treatment. This improvement in clustering is also seen in Figure 5.12 *(bottom*), where clusters exist for the 40-min and 1-h treatments, although there still remains some mixing of the spectra in the remaining clusters. The PCA analysis shows similar improvements upon selecting a smaller spectral

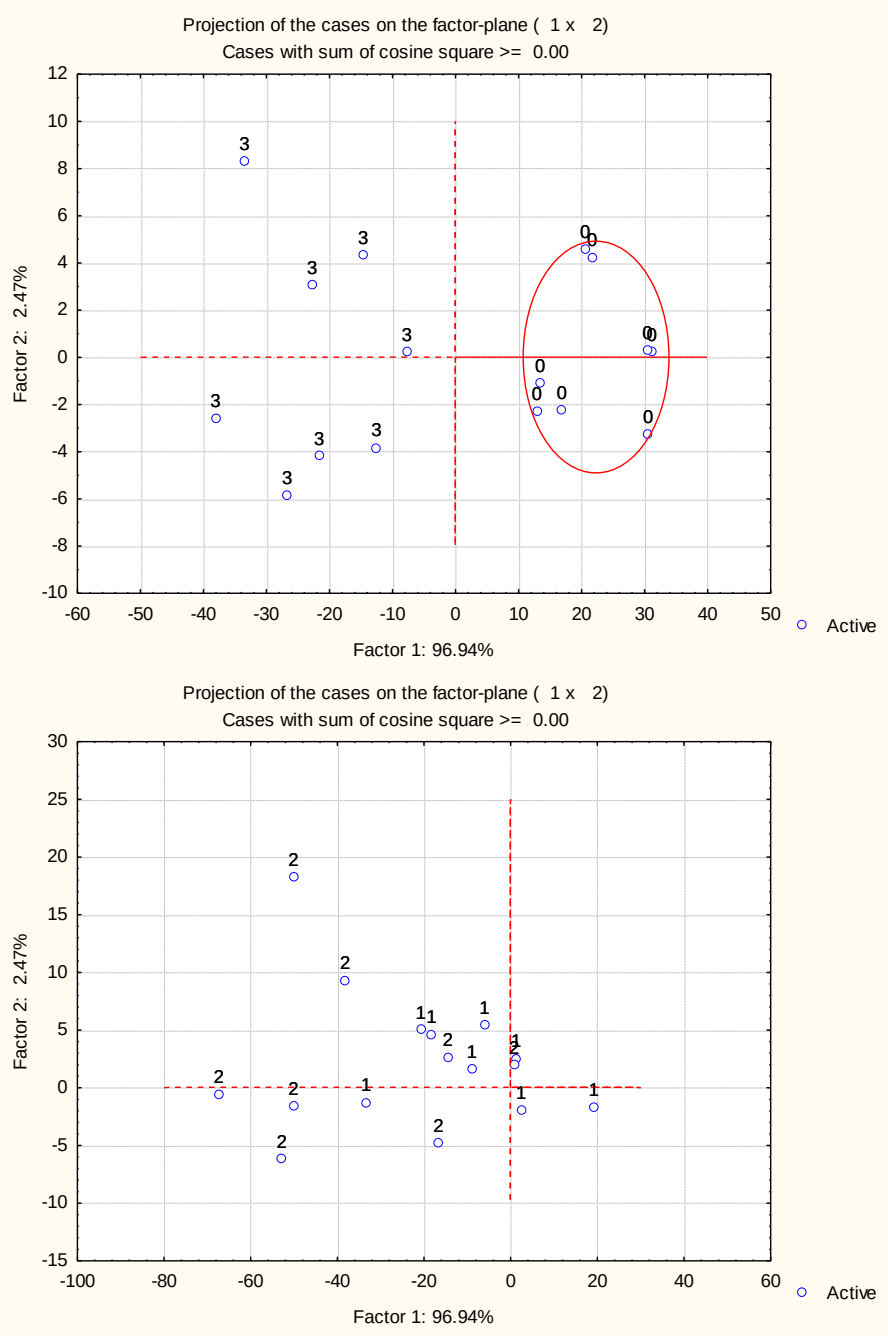

Figure 5.7. PCA analysis of DRIFT based spectra (2000-800 cm^{-1}) obtained from the raw and the autoclaved flaxseed samples. Comparisons of the separate treatments. .(*top)* 0 vs. 3, *(bottom)* 1 vs. 2 (0 = control/raw, 1 = autoclaved @ 120°C for 20 min, 2 = autoclaved @ 120°C for 40 min, 3 = autoclaved @ 120°C for 60 min) [PCA = Principal components analysis, DRIFT = Diffuse Reflectance Infrared Fourier Transform]

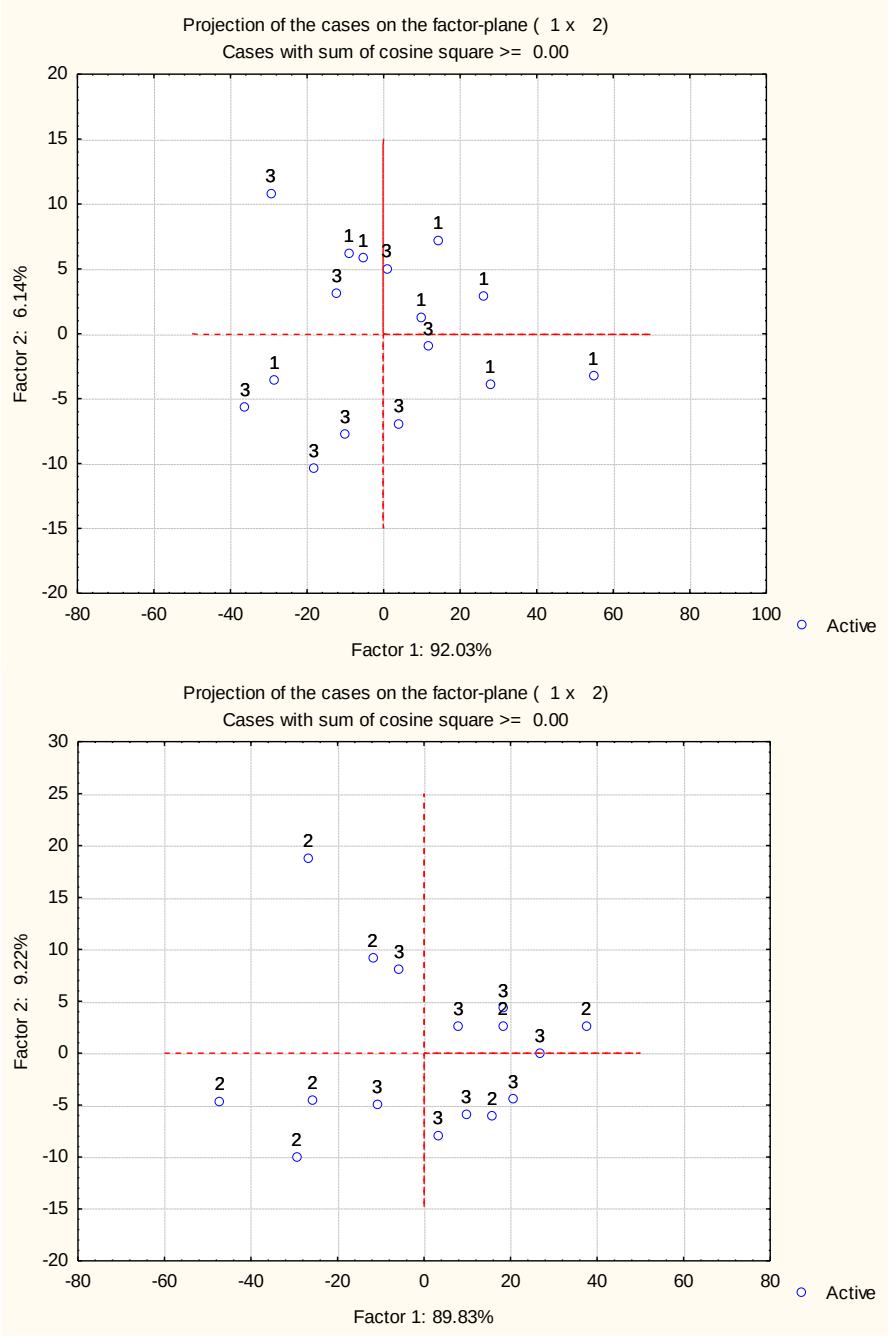

Figure 5.8. PCA analysis of DRIFT based spectra (2000-800 cm^{-1}) obtained from the raw and the autoclaved flaxseed samples. Comparisons of the separate treatments. .(*top)* 1 vs. 3, *(bottom)* 2 vs. 3 (0 = control/raw, 1 = autoclaved @ 120°C for 20 min, 2 = autoclaved @ 120°C for 40 min, 3 = autoclaved @ 120°C for 60 min) [PCA = Principal components analysis, DRIFT = Diffuse Reflectance Infrared Fourier Transform]

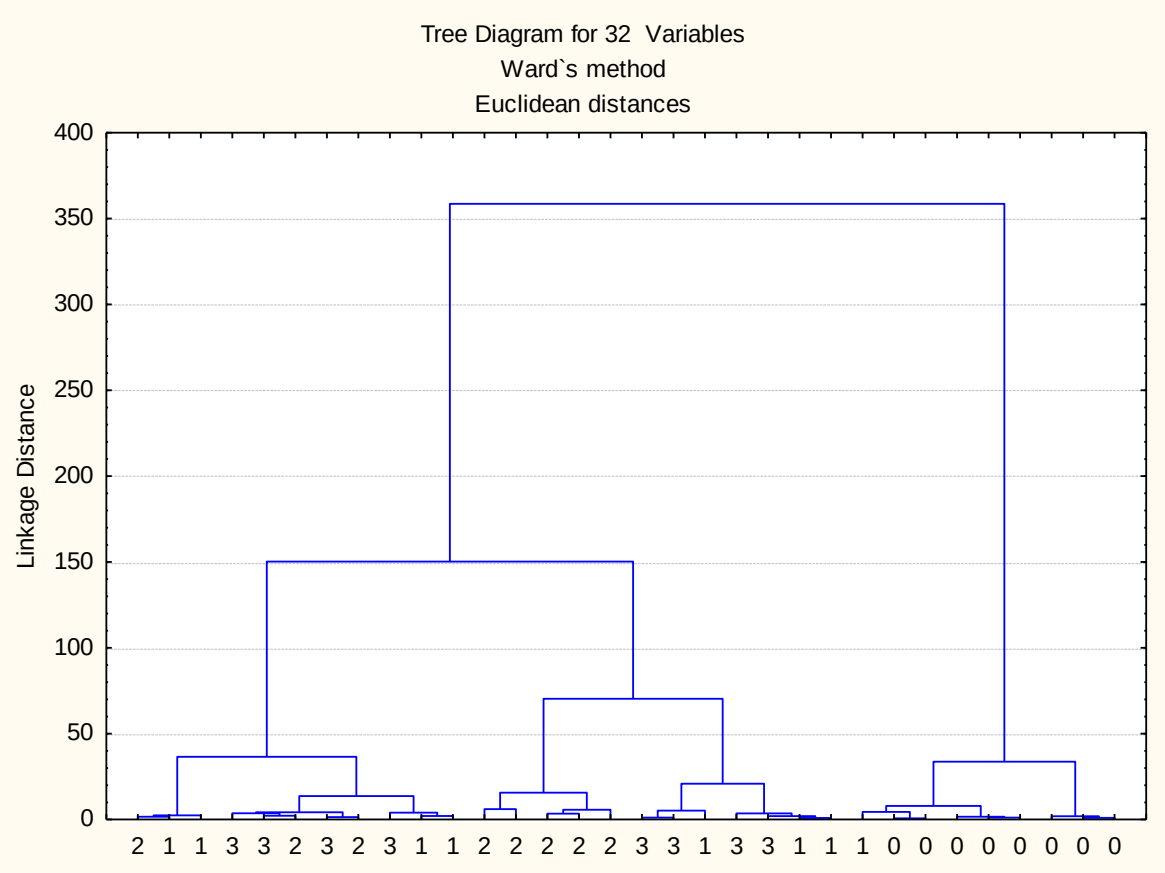

Figure 5.9. CLA analysis of DRIFT based spectra of the amide I and II region (1715-1485 cm^{-1}) obtained from the raw and the autoclaved flaxseed samples (0 = control/raw, 1 = autoclaved @ 120°C for 20 min, 2 = autoclaved @ 120°C for 40 min, 3 = autoclaved @ 120°C for 60 min) [CLA = Hierarchical cluster analysis, DRIFT = Diffuse Reflectance Infrared Fourier Transform]

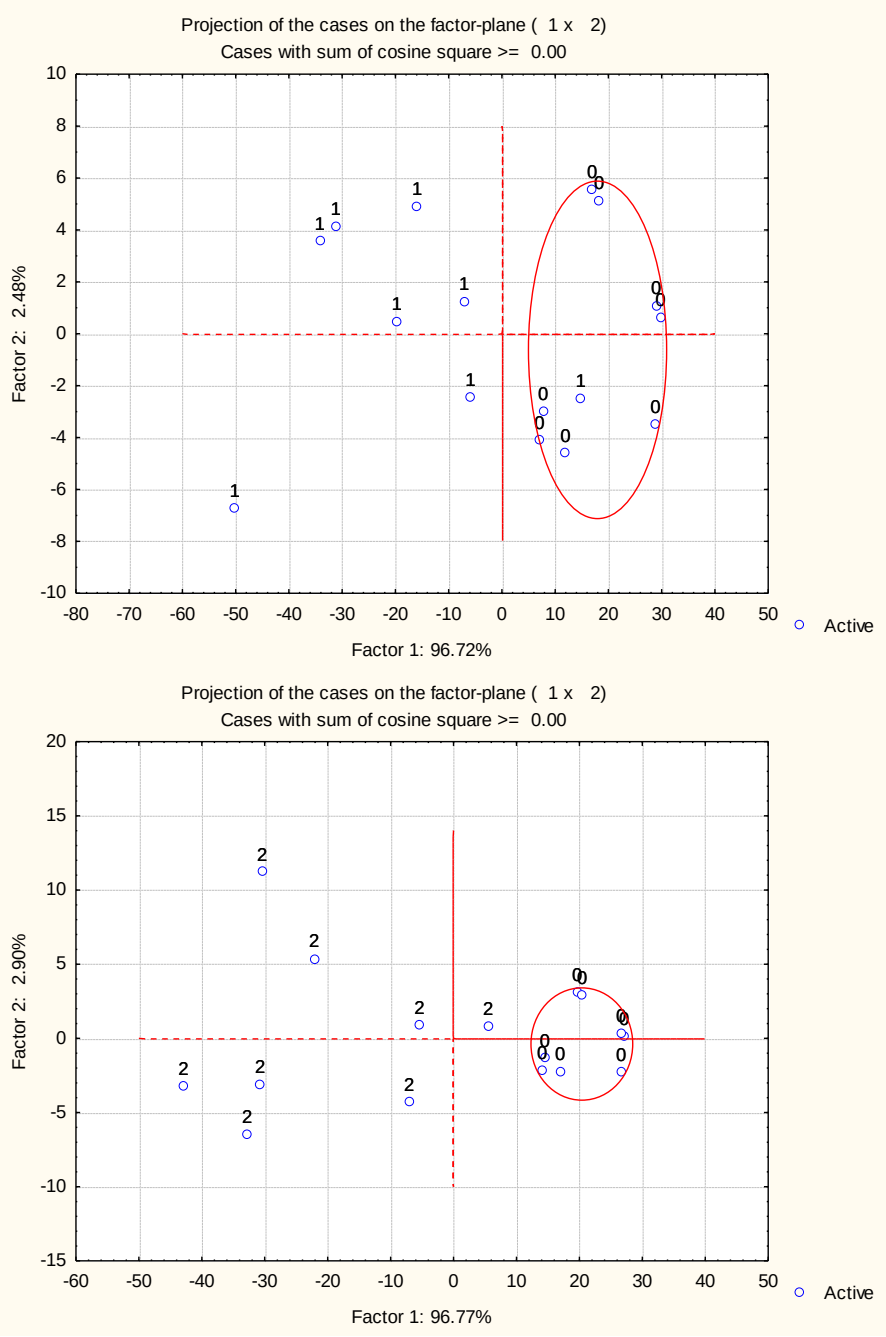

Figure 5.10. CLA analysis of DRIFT based spectra of the amide I and II region (1715-1485 cm^{-1}) obtained from the raw and the autoclaved flaxseed samples. Comparisons of the separate treatments.(*top)* 0 vs. 1, *(bottom)* 0 vs. 2 (0 = control/raw, 1 = autoclaved @ 120°C for 20 min, 2 = autoclaved @ 120°C for 40 min, 3 = autoclaved @ 120°C for 60 min) [CLA = Hierarchical cluster analysis, DRIFT = Diffuse Reflectance Infrared Fourier Transform].

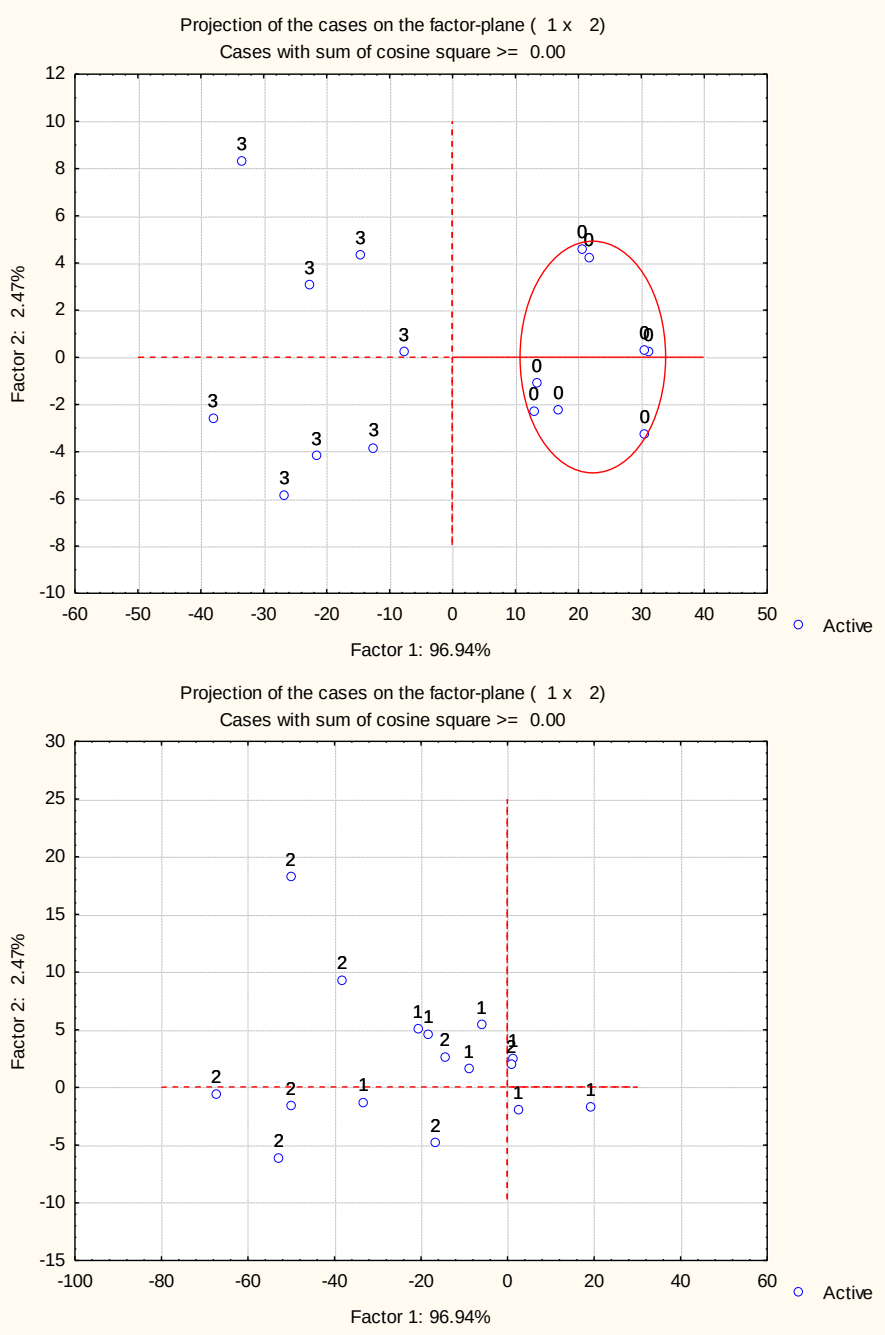

Figure 5.11. CLA analysis of DRIFT based spectra of the amide I and II region (1715-1485 cm^{-1}) obtained from the raw and the autoclaved flaxseed samples. Comparisons of the separate treatments.(*top)* 0 vs. 3, *(bottom)* 1 vs. 2 (0 = control/raw, 1 = autoclaved @ 120°C for 20 min, 2 = autoclaved @ 120°C for 40 min, 3 = autoclaved @ 120°C for 60 min) [CLA = Hierarchical cluster analysis, DRIFT = Diffuse Reflectance Infrared Fourier Transform].

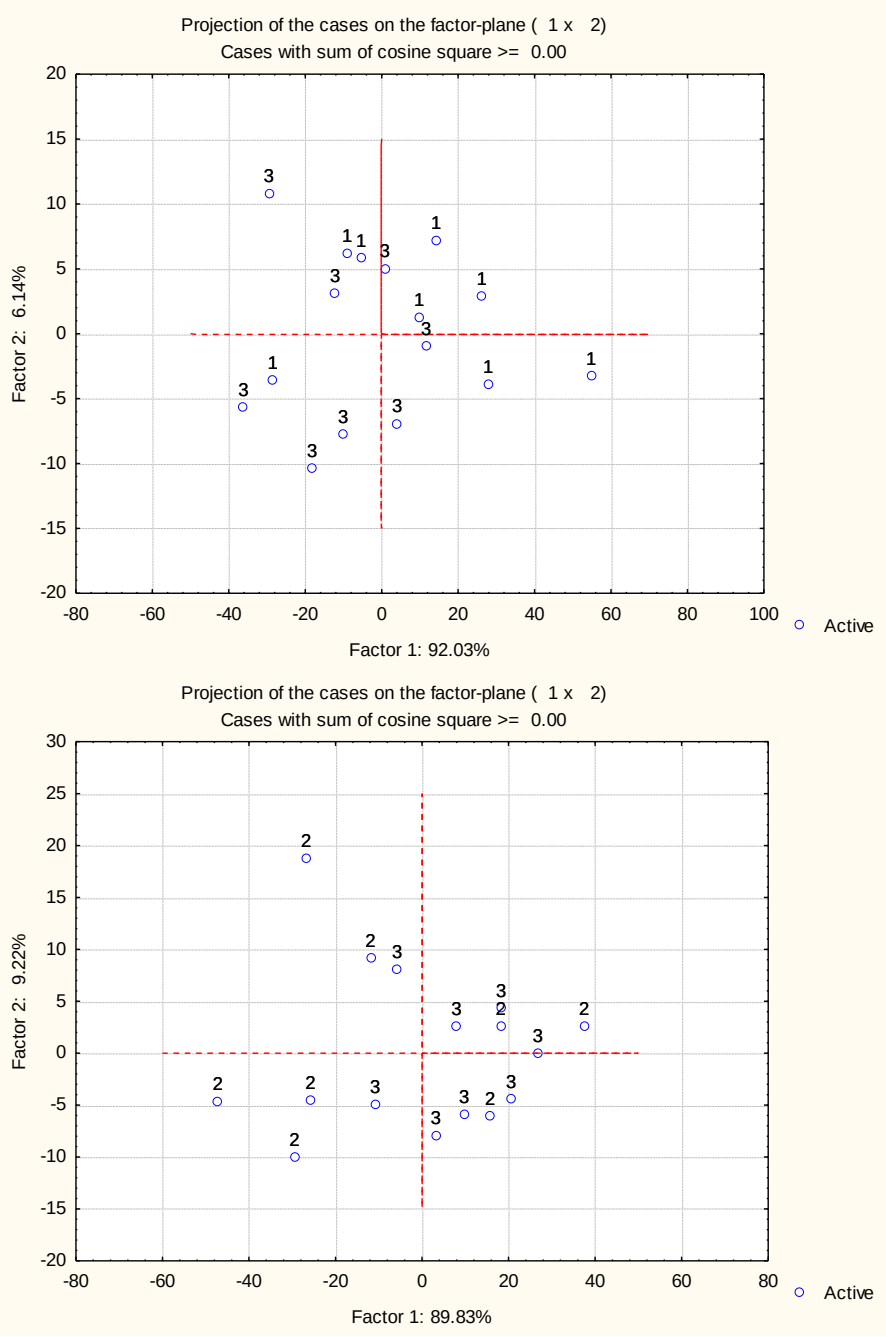

Figure 5.12. CLA analysis of DRIFT based spectra of the amide I and II region (1715-1485 cm^{-1}) obtained from the raw and the autoclaved flaxseed samples. Comparisons of the separate treatments.(*top)* 1 vs. 3, *(bottom)* 2 vs. 3 (0 = control/raw, 1 = autoclaved @ 120°C for 20 min, 2 = autoclaved @ 120°C for 40 min, 3 = autoclaved @ 120°C for 60 min) [CLA = Hierarchical cluster analysis, DRIFT = Diffuse Reflectance Infrared Fourier Transform].

window. In Figure 5.13, where all spectra are included in a PCA, there is no longer a spectrum from the 20-min treatment group present amongst the control sample spectra. Upon further examination of the comparisons between the treatments, shown in figure 5.14 *(top)*, *(bottom*) and Figure 5.15, it is apparent that autoclaved treatments are completely separated from the raw spectra. The separation indicated that treatment spectra differ from the raw spectra and suggested that there was a molecular structure change in the protein based on the amide I and amide II regions.

Although more research is required to establish how protein secondary structures can be used to forecast the degradability of a feed, changes to these structures are the result of autoclave treatment and cooking in general (Arnoldie 2001), which will influence degradability. It may be that data on the two primary secondary structures need to be supplemented with information about other secondary structures such as random coils to use this measure more accurately in predicting degradability. It is also evident that there needs to be a standardized and well controlled procedure for sample preparation as demonstrated by the difference in the relative ratios of α-helices to β-sheets as measured by multiple techniques using both DRIFT and S-FTIR.

It could be erroneous to rely solely on protein secondary structure ratios to predict digestibility. It has been shown that feathers are poorly degradable protein sources and are rich in β-sheets (Yu et al. 2004b). This, however, should not preclude feeds that are low in α-helices and high in β-sheets from being rapidly degraded in the rumen. The β-keratins that make up the feather have other molecular properties rendering them insoluble and poorly degradable, such as a high concentration of disulfide bonds. These

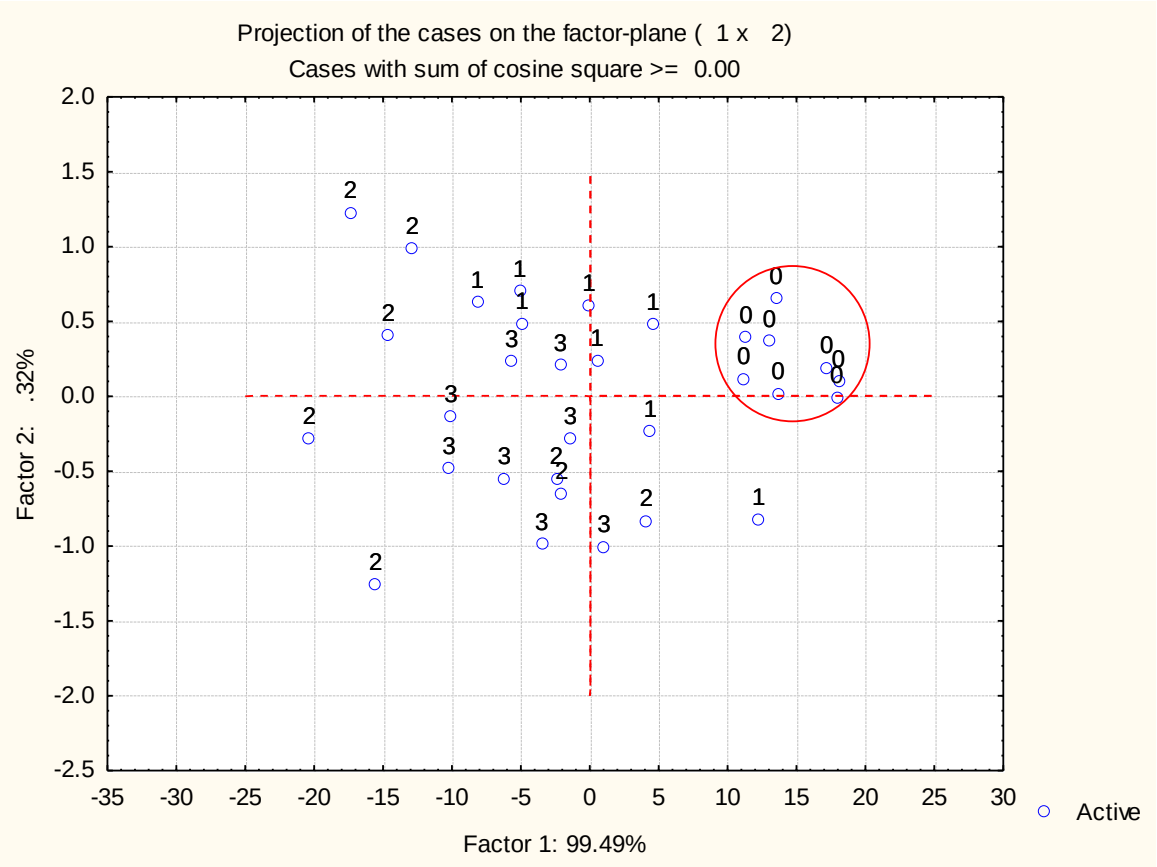

Figure 5.13. PCA analysis of DRIFT based spectra for the amide I and II region (1715-1485 cm^{-1}) obtained from the raw and the autoclaved flaxseed samples (0 = control/raw, 1 = autoclaved @ 120°C for 20 min, 2 = autoclaved @ 120°C for 40 min, 3 = autoclaved @ 120°C for 60 min) [PCA = Principal components analysis, DRIFT = Diffuse Reflectance Infrared Fourier Transform]

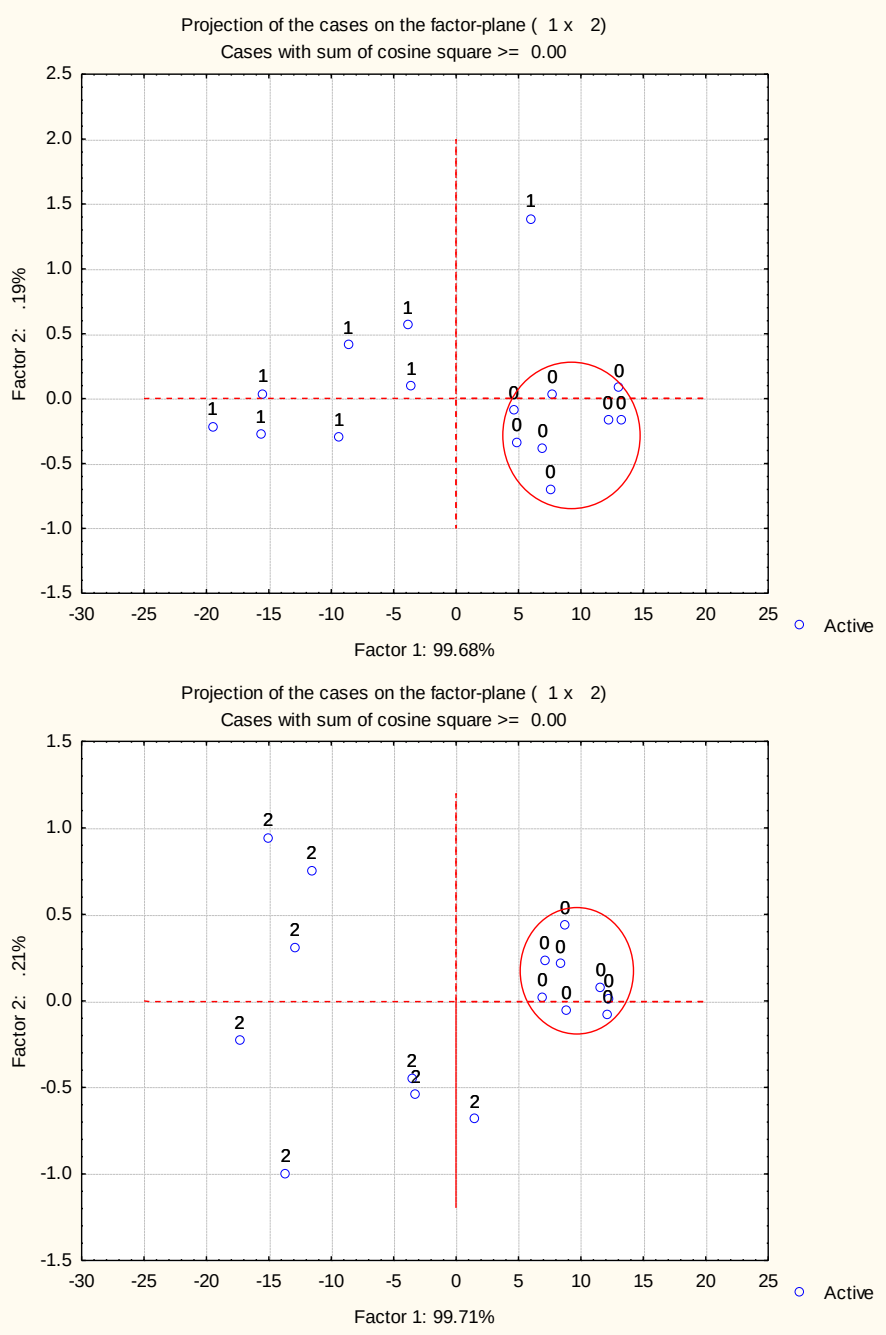

Figure 5.14. PCA analysis of DRIFT based spectra for the amide I and II region (1715-1485 cm^{-1}) obtained from the raw and the autoclaved flaxseed samples. Comparisons of the separate treatments. (*top)* 0 vs. 1, *(bottom)* 0 vs. 2 (0 = control/raw, 1 = autoclaved @ 120°C for 20 min, 2 = autoclaved @ 120°C for 40 min, 3 = autoclaved @ 120°C for 60 min) [PCA = Principal components analysis, DRIFT = Diffuse Reflectance Infrared Fourier Transform].

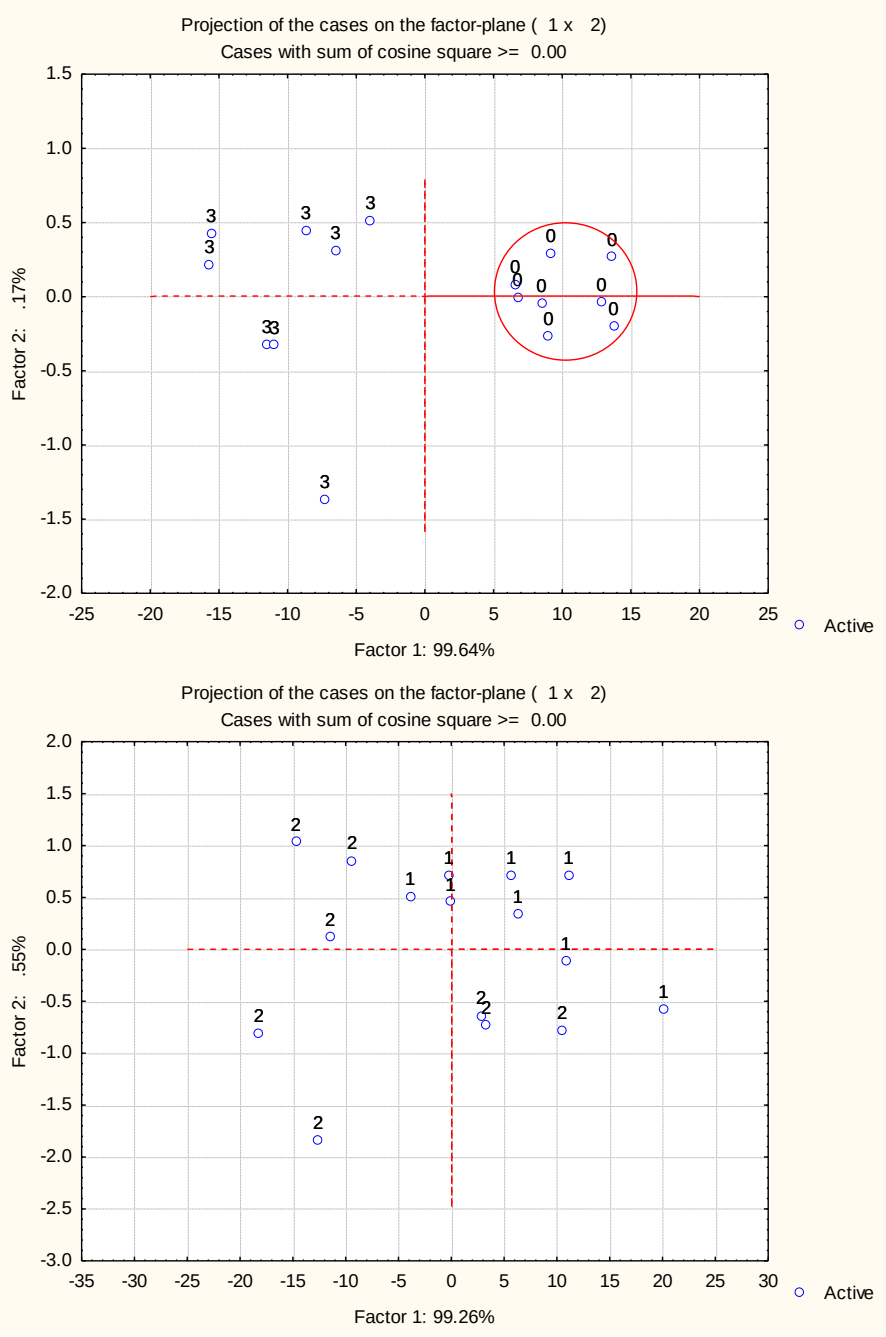

Figure 5.15. PCA analysis of DRIFT based spectra for the amide I and II region (1715-1485 cm^{-1}) obtained from the raw and the autoclaved flaxseed samples. Comparisons of the separate treatments.(*top)* 0 vs. 3, *(bottom)* 1 vs. 2 (0 = control/raw, 1 = autoclaved @ 120°C for 20 min, 2 = autoclaved @ 120°C for 40 min, 3 = autoclaved @ 120°C for 60 min) [PCA = Principal components analysis, DRIFT = Diffuse Reflectance Infrared Fourier Transform].

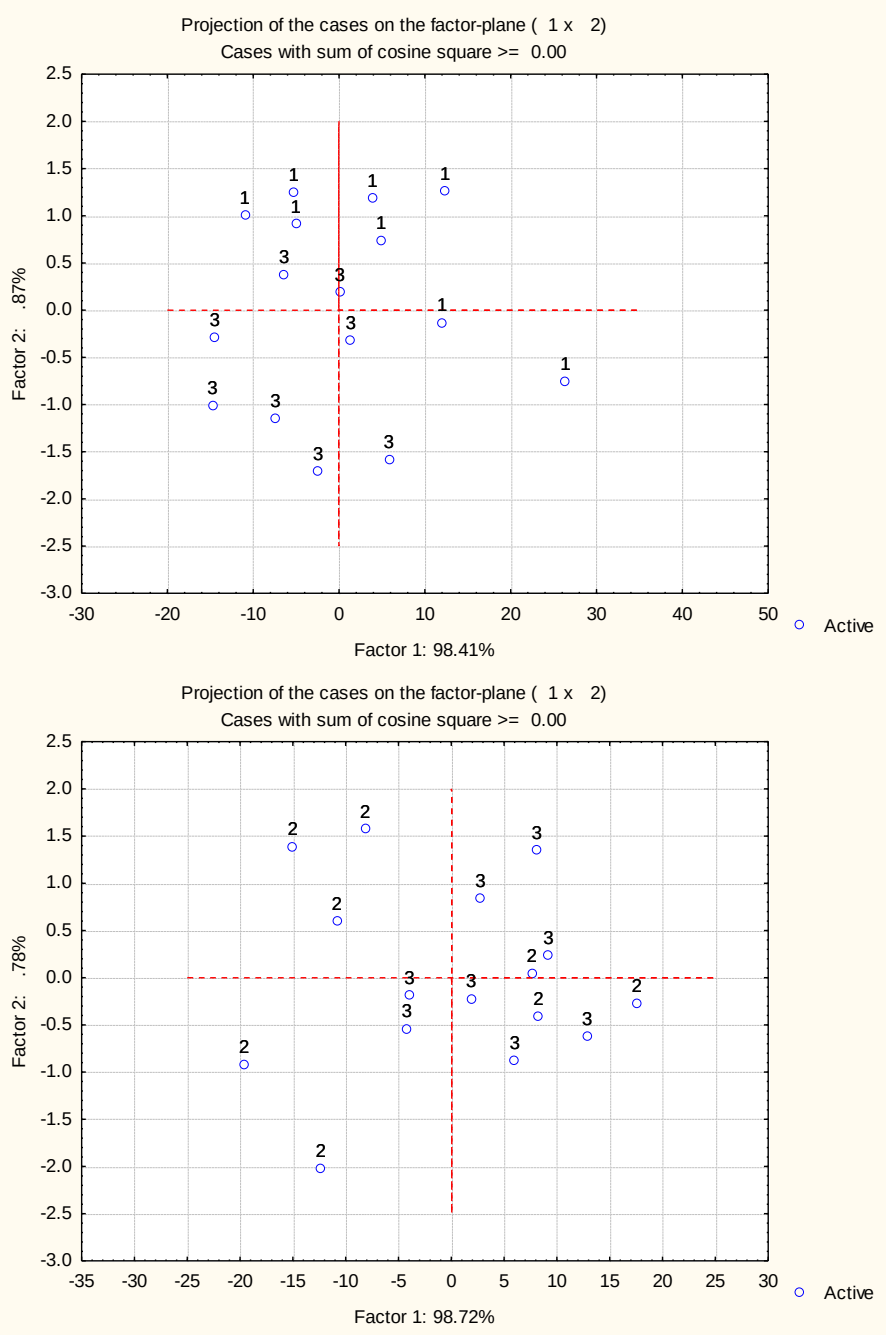

Figure 5.16. PCA analysis of DRIFT based spectra for the amide I and II region (1715-1485 cm^{-1}) obtained from the raw and the autoclaved flaxseed samples. Comparisons of the separate treatments.(*top)* 1 vs. 3, *(bottom)* 2 vs. 3 (0 = control/raw, 1 = autoclaved @ 120°C for 20 min, 2 = autoclaved @ 120°C for 40 min, 3 = autoclaved @ 120°C for 60 min) [PCA = Principal components analysis, DRIFT = Diffuse Reflectance Infrared Fourier Transform].

other features may have serious implications for degradability. Hair is made up of α-keratin which is high in α-helices and cysteine bonds. Hair, like feathers, has been shown to be poorly degraded as well (Thomas and Beeson, 1977). The degradability of keratin can be improved with NaOH treatment through the hydrolysis of cysteine (Stoves 1942), but these bonds are involved in tertiary and quaternary structure of keratin which are responsible for its lack of solubility. Further investigation into the digestibility and molecular chemistry of these two poorly degraded proteins may provide some insight into how secondary structure influences degradability.

Comparisons of protein secondary structure ratios for other feed sources may only be applicable when dealing with a single variety within the same genus and species. Protein content could differ within the same variety as well. It has been documented that growing conditions and topography have the capacity to alter the protein content of plants (Kravchenko and Bullock, 2002). Changes to protein quality will be reflected by different amounts of the various storage proteins specific to each plant (Paek et al. 1997), which means that the initial α-helix to β-sheet ratio could be altered by these conditions and after processing may show shifts in secondary structures as a result.

The results from the multivariate statistical methods used in this study show that by focusing on specific regions of the spectrum, the ability to separate spectra based on treatment conditions can be enhanced. This is likely a result of eliminating some extraneous data that does not contribute to differentiating between the treatments or, in the case of autoclaving, data for peaks that are minimally influenced by the treatment. In IR, spectra a large region, found between ca. 2500-2000 cm^{-1}, is usually devoid of any peaks of interest, so removing data contained in regions such as these from the analysis is

justifiable. Selecting windows or regions of the spectra will also allow us to determine which regions, representing certain molecules, are more or less affected by a given treatment.

Very little modification of the spectra and thus very little time is required for multivariate analysis. The implications of this are that with a pool of spectra, adding an unknown spectrum of interest to the cluster analysis procedure would discover if the sample has been autoclaved or not, provided all other things remain equal, sample preparation for example. With the 20 min difference in treatment times, the ability to make some distinctions between the treatments is indicative of the potential of this methodology. These results are further validated by those reported by Yu et al. (2007) where using S-FTIR spectra, and also without prior modifications of the spectra, cluster analysis was able to distinguish between those spectra from various wheat structures such as the endosperm and the aleurone layer.

5.4 Conclusions

The results from secondary structure analysis are not clear cut and more investigation is required to establish why the results from DRIFT and S-FTIR differed. DRIFT and S-FTIR, however, can be used to assess changes to protein structure as a consequence of heating. The multivariate statistical methods employed in this experiment show that, in terms of classification, they can provide an idea of degradability through association to the treatment conditions. Linking these results to degradability itself, rather than to treatment conditions, might prove to be a more effective way of using these statistical tools for this sort comparison. This would require identifying the variables in the analysis

(individual spectra) based on their degradability as opposed to time of treatment. Similar FTIR methodologies have been used to identify bacteria in the past, classifying different strains of the same genus (Maquelin et al 2002), so there is untapped potential for FTIR, whether DRIFT or S-FTIR based spectroscopies are employed in the feed industry.

6. GENERAL DISCUSSION, CONCLUSIONS AND FUTURE RESEARCH

The present study illustrates the sensitivity of mid-IR spectroscopic techniques in detecting subtle changes in the chemistry of a feed. It has also demonstrated the ability to identify more subtle aspects of feed chemistry, such as molecular conformation. Changes to feed chemistry without a link to structure are easily identified through common assays. The conformation or shape of a particular molecule, however, cannot be assessed by these common methodologies. The ability to consider these two data types at one time is the advantage of IR spectroscopy. The major drawback of mid-IR spectroscopy in complex samples such as feeds is the resulting complexity of the spectra.

In the search for spectral features that are indicative or even predictive of changes to protein degradability a multi-step approach was taken. First, the same flaxseed samples were put through the same treatment for different periods of time to mitigate effects on the spectra from the seed variety. This restricted differences in the spectra and degradation characteristics to being a result of the time of the treatment. To deal with the variation that exists between individual flaxseeds, a nested experimental design was used for S-FTIR results. Simply evaluating differences in spectra, however, may not provide a full indication of differences in digestibility or degradability. Second, well established methods of evaluating feed protein degradation characteristics were used to ensure that degradation characteristics were changed and to what extent the change occurred. Third, different techniques of mid-IR spectroscopy, DRIFT and S-FTIR spot sampling in this case, were applied to the sample. With these spectra, several methods were applied to investigate for changes that could be associated with degradability. In this case protein

was the focus. One approach was to determine the relative content of protein secondary structures. The second approach was to apply CLA and PCA to the spectra, where initially a large region of the spectrum was used then a much smaller region was selected, covering the major bonds associated with the peptide bond of protein, the amide I and II bonds.

Confirmation that degradability had indeed been altered by the applied autoclave treatments was demonstrated by several methods. *In situ* data showed that the rate and extent of protein disappearance was reduced by the treatments. Fractioning protein according to the CNCPS also showed an increase in the more slowly degradable PB2 fraction at the expense of the more rapidly degraded PB1 fraction. Looking at the individual nitrogen fractions decreases in the SCP are offset by increases in NDIN and ADIN, offers even more insight into the changes in observed in degradability. From this part of the study, it has been shown that the chemistry of the flaxseed protein had been altered. This is explained by the physical or chemical properties of the protein that the CNCPS takes advantage of for the purposes of fractionation, such as solubility. Interestingly, each of these fractions is assigned a specific degradation rate in the rumen. These types of changes in protein upon heating are common. An issue that arises from the data in the present study is the inability of these methods to distinguish one treatment from another, except in the case of the PB1 and PB2 values.

With the changes in degradation characteristics established, an attempt was made to identify changes to the spectra as a result of the sample treatment, which may be linked to degradability. Measuring relative amounts of α-helices and β-sheets is a means of assessing the change in the physical shape of a protein's inherent structure. Through the

process of denaturing, changes to the secondary structures are expected to some extent. The more denatured protein becomes, the greater the secondary structures should be affected. It's not unreasonable to expect that one secondary structure would be more susceptible to denaturation than the other, but which one is more susceptible to denaturation would be dependent on several factors specific to the particular protein or mix of proteins in question. The secondary structure ratio analysis was not always able to distinguish between raw and treated sample spectra. The DRIFT and S-FTIR results were not in agreement. The difference may be the result of different feed structures measured in each analysis. In the case of S-FTIR, only cotyledon measurements were taken, whereas DRIFT analyses were based on whole ground seed. This means the DRIFT spectroscopy results were based on a different complement of proteins than those of S-FTIR so this shouldn't be completely unexpected. It does, however, make it difficult to decide which results are more pertinent to degradability, if at all. Linking changes in secondary structure to shifts in CNCPS and other protein fractions as well as degradation characteristics may not be feasible. Changes in the various protein fractions are the result of protein binding to other feed constituents upon applying heat. In contrast, changes in secondary structure are likely the result of hydrogen bonds being broken. Both events occur concomitantly upon heating and are not necessarily dependant on one another. A potentially better target to link the changes in the protein fractions to IR spectroscopic data would be the bonds proteins form with fibre as a feed is heated. Focusing on the Maillard reaction with IR spectroscopy is another potential target that might be more revealing of protein changes linked to digestion. It is possible that there are too many protein specific factors for secondary structure analysis to be effective at predicting

degradability under varying conditions other than just time. It should be noted, however, that the degradation rate was not different for any of the treatments, which is reflected in the results from the multiple component peak modeling secondary structure analysis.

Multivariate methods permit the investigator to ignore many of the specifics when it comes to IR spectra. They allow us to identify associations or relationships among complex data sets without prior knowledge of the data in question. Generally speaking, the multivariate results used in this study showed most clearly the differences between raw and cooked flaxseed samples, with the 1-h treatment being the most readily identified as different from the control treatment. What was demonstrated in this study is that focusing the area probed by these methods, enhances their performance with respect to identification of treatment conditions. The analyses showed modest improvements in isolating different treatments based on just selecting the region of the IR spectrum where protein-specific bonds vibrate. This is due to the removal of data that is not associated with protein. The larger regions of the mid-IR spectrum used for multivariate analyses can include the bonds that are broken and formed during heating, but which are not considered in secondary structure analysis. The spectral data about these bonds would affect the results of multivariate analyses. There is a lot of potential in this kind of analysis for identifying those spectral windows that are indicative of degradability. Narrowing the spectral windows further can permit the identification of the IR wavelengths associated with degradability. Once known, these smaller windows could allow backtracking to the bonds behind observed changes.

The multivariate results from mid-IR spectroscopy appear to reflect some of the results from the companion study, in particular the results from the amide I and II regions.

Consider the SCP and CNCPS PB2 results compared with the cluster analysis results from the amide I and II spectral region. The 40-min and 1-h treatment spectra are clustered entirely on their own when compared to the control spectra. The SCP and CNCPS PB2 results for these treatments were not significantly different. The 20-min treatment has its own cluster, with the exception of one spectrum mixed among the control sample spectra. The SCP and CNCPS PB2 results show this treatment to be significantly different from all the other treatments. When all spectra are analyzed simultaneously, however, the relationship appears to fall apart as the only group of spectra that is almost entirely grouped on its own is the control group. The remaining spectra are in mixed clusters. It is evident that more research will be required to use multivariate spectral analysis for predicting protein degradability reliably.

There exists great untapped potential for mid-IR spectroscopy in the feed industry. It is clear from the spectral analyses conducted in this study that much work needs to be done to identify those features and regions of the spectrum that are most closely associated with changes in protein degradability. Using treatment in lieu of measured degradability as a basis for CLA and PCA obscures the fact that these were grouped accordingly, even using large regions of the spectra. Where PCA and CLA really shine was in their ability to show differences between the treatments in many of the comparisons, suggesting greater sensitivity to chemical methods.

The basic purpose for this study was to establish the manner in which mid-IR spectroscopic data can be used to predict how a feed and its components will be digested. The overall goal is to develop a means of rapid and accurate feed evaluation which could

not only save money but a lot of time. To achieve this, there are a few ways the problem could be approached with the spectroscopic data on hand. These include:

1. Using mathematical means such as the second derivative function to enhance differences between the spectra prior to PCA and CLA. This might enhance the ability of these multivariate methods to distinguish between the treatments by enhancing the variability between spectra.
2. Decreasing the size of that portion of the spectrum used for PCA and CLA. Using smaller-sized regions permits one to focus on the effects on a single bond and evaluating which bonds are better for differentiating between the treatments.
3. Including more than just the two amide I peaks representing α-helices and β-sheets in the spectroscopic analysis of protein. These major secondary structures are degraded somewhat upon heating and can result in other protein structures that were left out in the present study. The changes to these other peaks may be more sensitive to degradability. These other peaks might also represent the binding of protein to other cellular constituents.
4. Using Fourier self deconvolution or second derivative function on amide II bands, as well as analyzing areas under those peaks. There may be component bands there that more aptly reflect changes to protein. It should also be noted that despite the potential complexity of this band, it contains the peptide bond, unlike amide I which represents the carbonyl group in a peptide molecule.
5. Applying discriminant analysis to spectral data may provide some insight into those spectral features which are responsible for observed degradation

characteristics. This could allow identification of specific changes in the spectra that are associated with changes to degradability as opposed to treatment.

6. Creating a database with the existing data to apply towards future work. Using existing spectra for comparison and identification of degradation behaviour, PCA and CLA can provide one means for grouping spectra of unknown degradability to those with known degradability. This could be taken further to include more complex methods such as artificial neural network analysis for spectral identification once a sufficiently large database is created.
7. Evaluate the sensitivity each tissue may have as a response to heating. The endosperm, for example, may be comprised of different proteins which may respond differently than those found in the cotyledon of the seed. This may provide us with more insight as to why different results were observed in the α-helix to β-sheet ratios for the different spectroscopic methods.
8. A more extreme heat treatment that causes protein damage and accompanying changes to intestinal digestibility as well as spectral changes. This would provide spectra that actually contain elements from damaged protein with effects on *in vitro* intestinal digestibility and would permit the investigation of damaged proteins compared to those that are not damaged.

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8. APPENDIX

8.1 Additional data from *in vitro in situ* nutritional characterization study

Appendix Table 8.1. CNCPS Carbohydrate Fractions of the raw (control) compared to the autoclave treated Vimy flaxseed.

		Autoclave Treatment (120ºC)			
Fraction	Control	20 min	40 min	60 min	SEM
CHO %DM	30.4[a]	28.7[b]	28.1[b]	28.5[b]	0.35
NSC %CHO	56.0[b]	65.5[a]	66.1[a]	69.1[a]	1.60
CA %CHO	55.0[b]	64.4[a]	65.2[a]	68.0[a]	1.56
CB1 %CHO	1.0	1.1	0.9	1.1	0.1
CB2 %CHO	26.4[a]	14.7[b]	11.3[c]	6.9[d]	0.68
CC %CHO	17.6[b]	19.8[ab]	22.6[ab]	24.0[a]	1.40

[a, b, c, d] Means with same superscripts in the same row are not significantly different ($P < 0.05$) Means separated using the LSD method

SEM = Standard error of mean

Appendix Table 8.2. NRC 2001 Truly Digestible Fractions of the raw (control) compared to the autoclave treated Vimy flaxseed.

		Autoclave Treatment (120ºC)			
Component	Control	20 min	40 min	60 min	SEM
tdNFC	16.9	18.4	18.2	19.3	0.68
tdCPc	23.7	23.4	23.5	23.5	0.13
tdFA	41.1[b]	43.1[a]	43.5[a]	43.2[a]	0.34
tdNDF	5.8[a]	3.5[b]	3.0[c]	2.4[d]	0.12

[a, b, c, d] Means with same superscripts in the same row are not significantly different ($P < 0.05$) Means separated using the LSD method. (SEM = Standard error of mean, NFC = non fermentable CHO, CPc = crude protein , FA = fatty acids, NDF = neutral detergent fibre)

Appendix Table 8.3. Total Digestible Nutrients, Digestible, Net and Metabolizable Energy Estimates From NRC Dairy 2001 of the raw (control) compared to the autoclave treated Vimy flaxseed.

		Autoclave Treatment (120ºC)			
Estimates	Control	20 min	40 min	60 min	SEM
TDN1x	131.7[b]	135.3[a]	135.7[a]	136.4[a]	0.19
DE1x	5.8[b]	6.0[a]	6.0[a]	6.0[a]	0.01
DE3x	5.4[b]	5.5[a]	5.5[a]	5.5[a]	0.01
ME3x	5.2[b]	5.3[a]	5.3[a]	5.3[a]	0.01
NE3x	3.7[b]	3.8[a]	3.8[a]	3.8[a]	0.01
DE4x	5.1[b]	5.3[a]	5.3[a]	5.3[a]	0.01
ME4x	4.9[b]	5.0[a]	5.1[a]	5.1[a]	0.01
NE4x	3.5[b]	3.6[a]	3.7[a]	3.7[a]	0.01

[a, b, c, d] Means with same superscripts in the same row are not significantly different ($P < 0.05$) Means separated using the LSD method

(SEM = Standard error of mean, TDN = Total digestible nutrients, DE = Digestible energy, ME = Metabolizable energy, NE= Net energy)

Appendix Table 8.4. *In Situ* Degradation Parameters of Neutral Detergent Fibre of the raw (control) compared to the autoclave treated Vimy flaxseed.

		Autoclave Treatment (120ºC)			
	Control	20 min	40 min	60 min	SEM
K_d (%/h)	5.8[a]	3.8[b]	4.3[b]	5.2[ab]	0.46
T0 (h)	0.7[ab]	0.2[b]	0.5[ab]	1.5[a]	0.54
S (%NDF)	5.3	0	0	0	2.32
U (%NDF)	27.8[b]	28.8[b]	35.9[a]	36.6[a]	1.50
D (%NDF)	66.9	71.2	64.1	63.4	2.98
EDNDF (%NDF)	37.8[a]	27.6[b]	26.5[b]	28.7[b]	1.62
BNDF (%NDF)	62.2[b]	72.4[a]	73.6[a]	71.3[a]	1.62
EDNDF (%DM)	6.5[a]	3.9[b]	3.7[b]	4.2[b]	0.24
BNDF (%DM)	10.7	10.2	10.2	10.3	0.35

[a, b, c, d] Means with same superscripts in the same row are not significantly different ($P < 0.05$) Means separated using the LSD method

(SEM = Standard error of mean, K_d = degradation rate, T0 = lag time, S = soluble fraction, U = undegradable fraction, D = degradable fraction, EDNDF = effective degradability of neutral detergent fibre, BNDF = bypass neutral detergent fibre)

Appendix Table 8.5. In Situ Degradation Parameters of Ether Extract of the raw (control) compared to the autoclave treated Vimy flaxseed.

		Autoclave Treatment (120ºC)			
	Control	20 min	40 min	60 min	SEM
K_d (%/h)	19.2	14.9	21.4	23.6	6.53
T0 (h)	1.2^{ab}	1.6^{a}	0.4^{b}	0.4^{b}	0.49
S (%EE)	23.8^{ab}	17.8^{b}	21.9^{ab}	28.3^{a}	3.03
U (%EE)	8.7^{b}	10.5^{b}	12.6^{a}	12.5^{a}	1.34
D (%EE)	67.4^{ab}	71.7^{a}	65.5^{ab}	59.2^{b}	3.22
EDEE (%EE)	74.5^{a}	68.7^{b}	72.0^{a}	72.9^{a}	2.19
BEE(%EE)	25.5^{b}	31.3^{a}	28.0^{b}	27.1^{b}	2.19
EDEE (%DM)	31.4^{ab}	30.3^{b}	32.1^{a}	32.3^{a}	0.96
BEE (%DM)	10.7^{c}	13.8^{a}	12.5^{ab}	12.0^{bc}	0.96

[a, b, c, d] Means with same superscripts in the same row are not significantly different ($P < 0.05$) Means separated using the LSD method

(SEM = Standard error of mean, K_d = degradation rate, T0 = lag time, S = soluble fraction, U = undegradable fraction, D = degradable fraction, EDEE = effective degradability of ether extract, BEE= bypass ether extract)